

How not to sell public assets

Governments everywhere, notably in Eastern Europe, are bent on selling public assets to private investors. They have a lot to learn from the muddled sale of the British electricity industry, now under way.

THE British government is making a thorough muddle of its scheme for selling the nationalized electric power industry. So much has been clear for at least the past two years, since the publication of the original plan due to Mr Cecil Parkinson, the then Secretary of State for Energy. In its heyday, in the 1960s for example, the nationalized electricity industry was both the largest industrial enterprise in Britain and a monopoly as well. (People's rights to generate their own electricity were tightly circumscribed.) But the industry was big enough to be technically adventurous — it was a worldwide pioneer in the construction of large generating sets and, briefly, of nuclear power stations as well.

Administratively, the industry was organized into a dozen distribution companies (called area boards) and a generating organization called the Central Electricity Generating Board (CEGB), one of whose principal achievements was the construction of an efficient means of shipping electricity in bulk from one place to another, which was called the National Grid. But strictly speaking, it is wrong to describe this as a British arrangement; Scotland had two separate public utilities for making electricity, while there were (and will remain) different arrangements for Northern Ireland.

Error

Parkinson's basic error was his decision that the dozen area boards due to be sold as separate companies, each of which is destined to be a regional monopoly with powers to generate its own electricity if it wishes, should between them own and manage the National Grid. Meanwhile, according to the original plan, there would be two distinct generating companies, one big and one small, to own and operate CEGB's generating plants. The companies are now known as National Power and PowerGen respectively. Nuclear power stations were to have been assigned to the larger of the companies, but harsh reality has forced the hapless government to acknowledge that these must remain in public ownership (as "Nuclear Electric"). This happened on the eve of Parkinson's transfer in July last year to the more humble post of Secretary of State for Transport.

By his own lights, Parkinson's objectives were as laudable as they could be; he merely sought to subject the electricity supply industry to the rigours of competition. Fondly but mistakenly, he believed that could be accom-

plished by breaking CEGB into two unequal halves each of which would be free to solicit the custom of the distribution companies for its own particular kind of electricity in competition with whatever third parties might choose to generate electricity under the new rules. What he overlooked was the certainty that this would turn the privatized generating companies into wasting assets. For the privatized distribution companies will ultimately generate their own electricity, if not on economic grounds then because of the wish to know more clearly where they stand. Under the new regime, the National Grid will eventually wither away. It is no surprise that the two generating quasicompanies have been talking down their own selling price. It is an incidental irony that both of them, having read the fine print in their draft licences, are now busily arranging to sell electric power cheaply to bulk industrial users.

Ironies

Two more substantial ironies are worth remarking. First, nobody has thought of taking an axe to that old British custom whereby the sole suppliers of electricity are also among the chief providers of equipment driven by electric power; it would need a clever bunch of regulators under the new regime to ensure that the new distribution companies do not use their *de facto* regional monopolies to cross-subsidize the sale of electrical appliances which, once sold, will have the effect of increasing the use of electricity.

The second and more important irony is that stock market conditions are not now propitious for the sale of huge enterprises like the British electricity supply industry. Selling off the distribution companies will be hard enough. National Power will strain the pockets of investors by itself. PowerGen would soak up what remaining liquidity the stock markets have to play with in the first part of next year. That is why, late in the day, the British government has now further departed from its original plan by listening to proposals from the Hanson Trust, a publicly quoted private company, to buy PowerGen for a single one-off payment of around £1,500 million. The convenience of such an arrangement is not in dispute, even though it conflicts with the government's objective of stimulating wider share ownership in Britain. But this development has stimulated an energetic competition between other potential buyers of PowerGen — the



company's own management for one and a consortium including a coal-mining trades union. The outcome is likely to be a hasty and unseemly auction of an important industrial asset in which taxpayers — the true owners — will be short-changed.

The question of how matters might have been differently arranged may now be academic at least as far as the British industry is concerned. But it was plainly from the start mistaken to suppose that splitting the national generating business into two would by itself create the competitiveness the industry needs; the decision that, in future, anybody who wishes to build and operate a generating plant may do so would have been a sufficient guarantee of that. But the mere notion that the generating companies should be sold off at a fixed time (next spring) has enabled potential purchasers to talk down the value of the assets on sale (which is why the nuclear generating plant was taken out of National Power). It would have been much wiser to have constrained the monopoly powers of both the distributors and the generators of electricity, to have left the National Grid with the generators and then to have sold shares in the companies piecemeal, as the markets were able to absorb them.

Lesson

What can would-be privatizers elsewhere learn from all this? The most immediate lesson is that the sale of public assets is never simple. The most conspicuous difficulty in Britain stems from the conflict between the need to sell assets successfully and the need to maximize the selling price, which has revenue implications. The present British government, which has a long list of further privatizations in its sights (coal mining and railways, for example) would be well advised to make the sale of public assets more a matter of routine. The occasional bouts of hoopla attending the sale of nationalized industries in the past ten years may have entertained (and enriched) the City of London, but the transfer of ownership of these important businesses could have been more profitably and efficiently arranged if the sale process had been more gradual.

Although circumstances in Eastern Europe are very different, some of the same considerations apply. The big difference is that most Eastern European public assets are internationally unprofitable, and are unlikely to excite the interest of either local or foreign investors. Events may well show that those assets cannot be sold at all, in which case they will eventually have to be allowed to collapse. But there is a danger that nationalized industries that are successful, the Skoda works in Czechoslovakia for example, will be sold too cheaply to overseas interests because the governments concerned are over-eager for outside management experience and even for guaranteed markets in the West. There, as in Britain, there is a case for selling these assets gradually, in such a way that their true value can be determined by the market on the basis of trading in a small proportion of their shares before the whole enterprise is put on the block. □

To go or not to go

The National Academy of Sciences finds China too much of a puzzle.

THE US National Academy of Sciences Committee on Human Rights seems determined to tie itself in knots over the question of whether scientists should accept invitations to visit the People's Republic of China. The academy is right to think it important that, given the Tiananmen Square incident and its aftermath, people should ask what they can do to express effectively their outrage. The question is a vexed one, but not as vexed as the academy tries to make it: but there must surely be a better way of coming to a conclusion than supplying pairs of answers, each negating the other.

At one time or another, the reader of "Considerations Regarding Individual Scientific Visits to the People's Republic of China" will learn that outside pressure, such as may be applied by refusing an invitation and protesting to the Chinese government, may meet "with a measure of success"; or, on the other hand, may "strengthen conservative hardliners and weaken moderate reformers . . .". Chinese professors are variously said to be "anxious to receive correspondence and visits from their foreign colleagues", and likely to see a visit as an expression of "an insensitivity" to their plight. Even the question of safety is plagued with contradiction; the ending of martial law "may help to reduce further tensions"; but, on the other hand, "new violence could follow". In a nutshell, the academy concludes, "there are persuasive arguments on both sides of the question."

What sort of conclusion might be offered? The academy seems to hesitate between two views; that scientists should either go and look for ways to protest about human rights abuses while they are there (and after they come back), or not go and write to the Chinese government to protest. But surely the academy can adopt a more hard-nosed approach than this? The obvious factor that has been left out of this equation is the opinion of the Chinese themselves. Not the opinion of the politicians currently in power in China that is, but that of the people who are at risk. During every period of disturbance in China since the revolution, it has always been academics, including scientists carrying out basic research without political implications, that have suffered the most. It was true at the end of the 'Hundred Flowers' movement in the late 1950s, and more so again during the Cultural Revolution when two-thirds of the staff of the Chinese Academy of Sciences were removed from their posts. If any group should have a say in the actions of colleagues abroad, surely it is the people who have most to lose, or gain, from foreign help. Would it be too simple a solution to the academy's dilemma to ask of visitors to China that they stop wrestling with their consciences and say to their hosts, "Tell me, what is the best thing I can do to help you?" □

AIDS programme faces donor fatigue

- WHO donations 30 per cent below target
- Crisis postponed by "creative accounting"

THE World Health Organization (WHO)'s high-profile AIDS programme faces a funding crisis, with pledges of donations for 1991 running at 30 per cent below the planned budget of \$100 million.

Michael Merson, director of WHO's Global Programme on AIDS (GPA) says the shortfall will "affect all aspects of the programme at a time when the epidemic is getting worse". Revised estimates of human immunodeficiency virus (HIV) infection released by WHO last month show that 8-10 million people worldwide now carry the virus, with infection in developing countries spreading rapidly.

GPA relies on donations from WHO member countries above their basic WHO subscriptions, and has grown from its inception in 1986 to be WHO's largest health programme. The programme is involved with many aspects of the fight against AIDS and spends a large proportion of its budget in developing countries.

But donations to GPA are now levelling off, when Merson believes action needs to be stepped up to keep pace with the epidemic. US donations, for example, grew from \$2 million in 1986 to about \$25 million in 1989. But the 1990 and the proposed 1991 donations have remained at this level. One of Merson's staff says that GPA has until now managed to postpone the crisis by some "creative accounting" — pooling two years' donations from some countries into a single year's budget.

GPA staff are now preparing two 1991 budgets, one for \$100 million, and a \$70 million version with many schemes pared back. These will be considered in November by the programme's management committee, made up of representatives from WHO member states. In the meantime, Merson hopes to persuade developed countries currently giving very little to the programme, to contribute more.

Merson says that GPA's funding problems are shared by other agencies. If developed countries' bilateral donations to combat AIDS in developing countries were growing, and other intergovernmental organizations had increased resources, then the budget shortfall at GPA would not be a problem. Ideally, GPA should be "the donor of the last resort", helping developing countries to establish sound AIDS programmes and "filling in the gaps" left by other donors.

But some experts on the AIDS epidemic in developing countries argue that ensuring the effective spending of existing funds is a more urgent concern

than simply expanding programmes such as GPA to keep pace with the epidemic. Tony Klouda, of the International Planned Parenthood Federation, which runs an AIDS programme funded partly by the UK Overseas Development Administration, says that "just ploughing money into AIDS programmes won't address the factors that cause the spread of HIV". He says that in India, a major factor in the current explosion of HIV infection (see *Nature* 346, 499; 9 August 1990) is the pressure on poor people to give blood for money. Aid should be aimed at solving such underlying problems.

Hilary Hughes, editor of the *AIDS action* newsletter, believes GPA should continue to forge links with non-governmental organizations (NGOs) in develop-

INSTITUT LAUE-LANGEVIN

Researchers irradiated

Paris

Two researchers and two technicians were slightly irradiated on Wednesday last week by a neutron beam while setting up an experiment at the Institut Laue Langevin (ILL), Grenoble.

Although an inquiry team has still to report, the scientists apparently believed that the beam was closed when a shutter was still open. The two researchers are thought to have received a dose of about 2 rem and the technicians much less. The annual dose accepted to be safe is 5 rem. They were all back at work the following day after medical examinations.

According to Peter Day, director of ILL, one of the researchers was within the beam for "about 20 minutes", the other for less time. The estimated 2 rem dose is, he said, "an upper limit, based on the assumption that the scientists were in the beam for the whole time". But, he explained, they were reorienting a metal sample for a small angle scattering experiment and were "likely to have been moving around".

"Fortunately", said Day, "the incident is not serious, but we are treating it as if it were because it ought not to be able to happen". The 'D17' instrument being used by the researchers has a low flux on the sample and uses a cold neutron beam. "If the same thing happened on some of the 30 other instruments, the consequences could be more serious."

Day explained that safety precautions at ILL include a system of visual and aud-

ible warnings. A number of countries' national AIDS programmes have little impact on the poor majority of their populations, she says, but NGOs typically work closely and effectively with local people.

GPA built up strong links with NGOs under its founding director, Jonathan Mann. But this policy was opposed by WHO director-general Hiroshi Nakajima, and was one of the sources of conflict between the two men that led to Mann's resignation earlier this year. Merson has stated in public his intention to continue cooperating with NGOs, but some observers fear that Nakajima's influence will limit this policy. **Peter Aldhous**

■ FACING AN EXPECTED 1-1.5 million people with HIV infection by the year 2,000 in Asia alone, representatives from 31 countries at the meeting on AIDS in Asia and the Pacific held in Canberra last week announced the creation of the AIDS Society for Asia and the Pacific (ASAP). The society will be modelled on the African Association for the Prevention of AIDS; it has support from GPA and will be seeking membership in the International AIDS Society.

Tania Ewing

ible warnings. Green and red lights indicate whether or not the neutron beam is on, while infrared 'trip' beams trigger an audible alarm if a person enters certain areas when it is not safe. It seems that last week these signals may have given contradictory information about the state of the instruments.

A researcher at the Rutherford Appleton Isis neutron scattering laboratory in Oxfordshire in the United Kingdom, who also works at ILL, said that ILL's safety record is generally "first rate", but added that it depends partly on the training and "professionalism" of full-time staff and is open to human error. At Isis, he explained, all the beams are interlocked and captive keys make it impossible to enter risk areas unless the beam shutters are closed (the same key has to be used either to open the shutter or to open a door).

"Our level of safety is absolute, the level at ILL is very safe", he said. But the Isis laboratory is both more recent and inherently more dangerous, using a spallation neutron source with energy up to 100 MeV, compared to the 1 MeV at ILL. "I've been in many laboratories around the world where safety is non-existent", he said, "but ILL is not one of them."

Peter Day expected the inquiry to report "within a day or two", with investigators currently looking at the shutter as a possible cause of the error. By an apparently unrelated coincidence, the post of safety officer at ILL is vacant.

Peter Coles

Lucky winners announced

Tokyo

AS in previous years, the biosciences and in particular molecular biology take by far the largest share of the "special distinguished" and "priority" grants announced two weeks ago by Japan's Ministry of Education, Science and Culture (MESC). But astrophysics and oceanography also feature prominently in the awards which are the biggest and most respected in MESC's grant repertoire.

The special distinguished grants, which go to individuals for "internationally recognized research that is likely to produce outstanding results", take effect immediately. The priority grants just announced, which support large teams of scientists, begin next fiscal year and their size is still subject to negotiations with the Ministry of Finance. Nevertheless, the amount of money awarded is usually fairly close to that requested.

The budget for priority grants has risen rapidly since they were introduced in 1986 and they now account for over a quarter (27 per cent) of MESC's annual research grant budget of about ¥50,000 million (\$340 million). Twenty-two grants were awarded in this category together with eleven special distinguished grants.

Minoru Kanehisa of Kyoto University's Institute for Chemical Research is set to get a large priority grant to develop computer techniques for analysing DNA
SUPERPHENIX

In trouble again

Paris

THE French 1,200 MW fast-breeder reactor, Superphénix, near the Swiss border, has been shut down again, this time because of oxidation of its liquid sodium coolant. The incident, which is rated '2' on a six-point risk scale for nuclear installations, is due to an influx of air into the cooling circuits, rendering the sodium more viscous and less efficient as a coolant.

The present shut-down, which is expected to last several months, means that the prototype reactor will have been operating for only just over half the time since it first came on stream in January 1986. In 1987 a leak in the reservoir containing the liquid sodium led to the reactor being shut down for more than 18 months. In both incidents, the reactor continued to operate for over two weeks before engineers were able to conclude from monitoring data that there was a serious fault. The government service for safety in nuclear installations (SCSIN), part of the industry ministry, has ordered an inquiry into the latest incident and expects to develop new recommendations on monitoring procedures.

Peter Coles

sequences of human and other genome data. Kanehisa is on an MESC committee that is planning to establish a human genome project in Japan in the next year or so, and his grant request of ¥850 million (\$5.7 million) over five years will give a significant boost to Japan's current small financial commitment to human genome research.

One of the biggest priority grants (a request for ¥1,180 million for three years) goes to Hideyuki Ogawa of Osaka University for a project intended to elucidate the molecular mechanisms of genetic recombination by focusing on how homologous DNA molecules recognize each other.

Many of the awards in the field of chemistry have biological themes. Tsujiaki Hata of the department of life chemistry at Tokyo Institute of Technology gets a priority grant with a request for ¥550 million over three years to develop new methods for the large-scale synthesis of oligonucleotides.

Kazuhiro Maruyama of the department of chemistry at Kyoto University wins a special distinguished grant of ¥200 million over four years to synthesize and characterize an artificial photosynthetic reaction centre. And Yukito Murakami of the department of organic synthesis at Kyushu University will receive ¥205 million in the same category for the molecular design of artificial enzyme systems.

The biggest special distinguished grant in biology (¥238 million for five years) goes to Katsuhiko Mikoshiba at the Institute for Protein Research at Osaka University for his pioneering research on the InsP_3 (inositol 1,4,5-trisphosphate) receptor in neurons and its role in neuronal signal transduction in the brain (see *Nature* **342**, 32; 1989). Takeyuki Wakabayashi of Tokyo University wins his second special distinguished grant, this time ¥199 million over four years, to continue his research on the molecular mechanism of muscle contraction using protein engineering and cryo-electron microscopy.

In oceanography, Asahiko Taira of the Ocean Research Institute at Tokyo University is awarded a special distinguished grant of ¥210 million for three years to produce high-resolution seismic images of the Izu-Ogasawara arc system. A team headed by his colleague Hitoshi Sakai at the same institute will examine ocean fluxes of terrestrial and biologically produced materials in the western North Pacific with a three-year priority grant provisionally set at ¥640 million.

Some of the largest grants go to astrophysics. Norio Kaifu of the National Astronomical Observatory heads a team that will set out over the next four years to

understand the elementary process, circulation and evolution of interstellar matter with a proposed priority grant of ¥970 million. Takashi Nakamura of the Yukawa Institute for Theoretical Physics at Kyoto University has requested a similar amount (¥930 million for four years) for his team to try to be the first to detect gravity waves, possibly from the formation of a black hole or neutron star. The biggest special distinguished grant of all (¥255 million for five years) goes to Kimio Niwa of Nagoya University, who will try to determine the mass of the tau neutrino.

Notable by its absence among the awards is cold fusion. Despite persistent rumours that Japan is carrying out a co-ordinated research effort in this field, MESC's only award for cold fusion research is a small grant of ¥10 million (\$87,000) to Hideo Ikegami of the National Institution for Fusion Science.

David Swinbanks

More grant awards

Tokyo

JAPAN'S Ministry of Education, Science and Culture (MESC) is no longer the only body to award big grants to university researchers.

Within days of MESC announcing its biggest grants, the New Energy and Industrial Technology Development Organization (NEDO), an organization affiliated with the Ministry of International Trade and Industry (MITI), awarded six large grants to international teams of university researchers for investigation of new materials. The NEDO grants and MITI's related Human Frontier Science Program represent a new and welcome source of money for Japan's fund-starved university researchers.

The NEDO grant programme was established in 1988 and awarded the first international grants for research on the brain and molecular biology under Japan's Human Frontier Science Program. Responsibility for Frontier grants has now been transferred to the Human Frontier Foundation in Strasbourg. But NEDO continues to award similar international grants for research on new materials.

Last week's awards, which are each worth about ¥30 million (\$200,000) a year and can be extended up to three years, go to six teams comprising 31 researchers from Japan (15), the United States (9), France (3), West Germany (3) and the United Kingdom (1).

At present the NEDO grants are restricted to material science but Masami Takayasu of NEDO says that NEDO is on the lookout for other suitable areas of research to support and would "welcome suggestions".

David Swinbanks

ANIMAL RIGHTS

Court loss may hurt activists

Washington

In a legal decision that could restrict the tactics of animal-rights investigators, a Las Vegas jury last week found two animal activist groups guilty of defamation and invasion of privacy for distributing a videotape allegedly portraying cruelty to animals.

The jury awarded \$4.1 million to Bobby Berosini, a Las Vegas showman whose act features trained orang utans. Berosini had sued People for the Ethical Treatment of Animals (PETA), America's largest animal rights group, and the Performing Animal Welfare Society (PAWS) for distributing videotapes that show Berosini striking the orang utans backstage with an instrument described as a metal rod.

The verdict could force PETA and other animal rights groups to restrict their distribution of evidence documenting animal abuse in research laboratories, according to PETA attorney Philip Hirschkop. Speaking last week, before the verdict was announced on 11 August, Hirschkop said: "If Berosini can pull this off, it will set animal rights protection back years. Groups will be terrified to go public with allegations." The jury found damages of \$3.1 million against PETA and \$1 million against PAWS for defamation, "intrusion into a seclusion" (the camera surreptitiously used to take the videotape had been placed in a private area backstage), invasion of privacy and misappropriation of name (Berosini's name had been used by PETA in articles about cruelty). During the trial, PETA officials and attorneys were fined a total of \$50,000 for giving a press conference and for publicly accusing District Court Judge Myron Leavitt of conflict of interest. (Leavitt has been a college roommate and law partner of an owner of the Stardust Hotel, where Berosini performs, and has received campaign contributions from the hotel. Leavitt ruled that there was no conflict.) Hirschkop says the groups will appeal against the jury's decision to the state supreme court. If they lose that case as well, they are prepared to appeal to the US Supreme Court, he says.

Dancers in Berosini's show had set up the camera to record what they suspected were regular backstage beatings of the orang utans.

They sent the tapes to PETA and PAWS, both of whom eventually redistributed it. It was shown on the US television programme "Entertainment Tonight". Although the tape is not narrated, PETA has solicited separate opinions from respected primate experts (including Jane Goodall) who found that the tape showed cruelty and gross abuse. Berosini testified that he was simply "correcting" the animals as part of their training.

PETA co-founder Alex Pacheco says that the decision will not stop PETA from distributing evidence of cruelty when it is found. But Hirschkop concedes that the decision is likely to have a "practical devastating effect" on animal-rights investigations.

Because the arguments in the case revolved around whether or not the tape depicted actual cruelty, the Las Vegas case may now impose a burden of legal proof on accusers before they make public allegations of cruelty. "If you're going to allege a fact, you're going to have to make sure that the fact is true", says Steven Wise, president of the Animal Legal

ELECTROMAGNETIC RISK

All aboard the bandwagon

Boston

Just a few months after the release of a highly controversial US Environmental Protection Agency (EPA) report* on the health effects of exposure to extremely low-frequency electromagnetic fields (EMF), the federal bandwagon is beginning to roll for more research on the effects of EMF. The EPA report claimed that existing evidence was sufficient to demonstrate a "significant" link between EMF exposure and human cancer (see *Nature* 345; 463; 7 June 1990).

At congressional hearings late last month, industry and government officials offered virtually unanimous endorsement of a proposed bill that would establish a coordinated, five-year, \$34-million federal research programme into the effects of electromagnetic fields. Among the bill's provisions are plans for a newly created "Electric and Magnetic Fields Research Advisory Committee" composed of researchers and representatives from government, industry and environmental organizations to recommend priorities and direction for the new research. The proposed bill is expected to go to the floor of Congress either this autumn or next session.

Support for a greater federal research effort marks a surprise change in direction for many participants involved in this issue. Robert San Martin, for example, who testified on behalf of the US Energy Department in favour of a new federal programme, had only several months earlier told Congress that the Energy Department considered its research on electromagnetic fields to be "satisfactory", rejecting suggestions that the agency might increase its effort. And sup-

Defence Fund. "The case may stop people from as quickly characterizing something as cruel." Although the decision is expected to encourage similar lawsuits from researchers who have been targeted by activists, the precedent may be psychological rather than legal. "That door was always open", says Barbara Rich of the National Associations for Biomedical Research. "Just because it wasn't taken, doesn't mean the cases couldn't have been won." And she points out that such lawsuits are still hugely expensive (PETA's legal fees are estimated at \$500,000) and tend to involve testimony damaging to both sides. "Most attorneys will tell you it's not worth doing unless you're on some sort of vendetta", she says.

Christopher Anderson

port for the federal programme came also from each of the major US electric-utility-backed groups.

Many questions remain. Observers differ, for example, about which agency should play the lead role in funding and conducting the research. As proposed, the congressional bill would give a primary role to the Energy Department, which currently spends roughly \$3 million annually on research in this area. But Louis Slesin of the New-York-based trade newsletter *Microwave News*, who has followed the issue closely for a decade, believes that the lead role should go to an agency "whose main job is public-health related". Slesin says he believes the Environmental is the most likely agency to oversee such a research project, but adds that he is "surprised" by its apparent "nonchalance" about the issue in recent years. In the past, the EPA sponsored research into the health effects of electromagnetic fields, but this was halted in 1986 during the Reagan Administration.

Questions also remain over whether a new federal effort should immediately include research into ways of reducing human exposure to electromagnetic fields and thereby mitigating their potential effects. While there is no decisive verdict on the biological effects of electromagnetic fields, Representative James Scheuer (Democrat, New York) who chaired the recent congressional hearings, stated that we "will have lost little" by initiating such efforts now even if no increased risk is found to be associated with exposure to EMF. Industry representatives, such as James Cunningham, vice-president of the New York Power Authority, however, foresees increased regulation which they believe has yet to be justified by the scientific evidence. As Cunningham put it: "What is needed now is not regulation, but research".

Seth Shulman

*Evaluation of Potential Electromagnetic Carcinogenicity, Office of Health and Environmental Assessment, US Environmental Protection Agency, 28 June, 1990 (EPA-600/6-90 005A).

Move to overturn ban

Washington

IN a move that is certain to revive an old controversy, Representative Henry Waxman (Democrat, California) has introduced legislation to overturn the present moratorium on the use of US federal funds to support fetal tissue research. The two-year-old ban covers the transplantation of human fetal tissue from induced abortions into human recipients, but does not extend to the use of fetal tissue for transplantation into animals or for use in other more basic research studies. Despite the ban, privately funded clinical trials of fetal-tissue transplants are continuing in patients with Parkinson's disease and diabetes, and many scientists believe that patients with Alzheimer's disease, epilepsy, Huntington's disease, spinal cord injury and AIDS could benefit from fetal tissue transplantation research.

In addition to lifting the ban, Waxman's bill, entitled "Research Freedom Act of 1990", would establish a mechanism for the review and approval of experiments in biomedical and behavioural research. Once an experiment has been reviewed and recommended for approval by both an institutional review board and a peer-review group, National Institutes of Health (NIH) funding could be denied on ethical grounds only on the recommendation of an ethics advisory board (EAB) of legal, ethical, religious and scientific experts convened by the Secretary of Health.

Douglas Johnson, legislative director of the National Right to Life Committee, opposes Waxman's initiative and says that it does much more than simply lift the ban. Under the proposed legislation, the secretary would be required to support any research approved by the EAB and, therefore, "would no longer have the final word", he says.

The ban was introduced temporarily in March 1988 by the then Assistant Secretary of Health Robert Windom after the NIH had sought his approval for an experiment involving the transplantation of human fetal cells into the brain of a patient with Parkinson's disease. Windom withheld approval pending the deliberations of an NIH panel set up to examine the ethical, legal and scientific implications of the experiment. The panel concluded that fetal tissue transplant research was "acceptable public policy" provided that certain safeguards were developed to prevent the commercialization of fetal tissue and to ensure that the decision to abort was made independently of the decision to donate fetal tissue to research.

Despite the panel's recommendations, Secretary of Health Louis Sullivan extended the ban indefinitely last November (see *Nature* **342**, 105; 1989). He argued

that women who are ambivalent about abortion may be influenced by the knowledge that the fetal tissue could be used to treat human disease, and concluded that the department should not be funding activities that encourage or promote abortion.

Curt Freed of the University of Colorado believes that the ban has considerably hampered research and adds that it is not just a question of money being available but also the "feeling that the government is not approving of this work and therefore maybe you shouldn't be doing it". Freed was the first US scientist to implant fetal neurons taken from the brain of a first-trimester aborted fetus into the brain of a patient with Parkinson's disease. The rationale was that fetal cells, which are thought to survive and integrate much better in the adult brain than transplants of more mature tissue, would produce

JAPANESE RESEARCH

MITI envious of MIT

Tokyo

JAPAN'S old habit of worrying just will not go away. Never mind that its economic miracle has made it the envy of the world, never mind that the Japanese live longer, never mind that their cities are the safest in the world, they still worry that they may be missing something. Now the Japanese have spotted that they do not have a world-class international centre of academic excellence — a Massachusetts Institute of Technology (MIT) or a Max-Planck Institute. Having discovered what is missing, the Ministry of International Trade and Industry (MITI) and the Science and Technology Agency (STA) are now worrying what to do about it.

Unfortunately, MIT is not for sale (yet). But leading foreign academics might be. One suggestion is that Japan should set up a new centre of excellence with some foreign staff, or a famous foreign director to give it class. (This is exactly what the Japanese did a little over a hundred years ago when they were setting up Tokyo University. Eventually many of the teachers were sent home — but while they were there their salaries were 3–4 times that of their Japanese colleagues.)

Concern over the missing centre of excellence first emerged back in 1988, in STA's annual white paper. Just to be sure that the concern was not unfounded, a survey followed. As predicted, it proved conclusively that most top Japanese academics have never sighted a centre of excellence in Japan (see *Nature* **344**, 94; 1990). The absence of such a centre was immediately put forward as one explanation for the failure of young foreign

dopamine, the chemical whose absence causes the uncontrolled tremors and muscle rigidity symptomatic of Parkinson's disease.

By contrast, Keith Crutcher, a neuroscientist at the University of Cincinnati who objects to most cases of induced abortion and who does not use human fetal tissue in his research, believes that "as scientists we cannot really divest ourselves from the need to look at the moral consequences of what we are doing".

Waxman's proposed legislation, which is supported by Ted Weiss (Democrat, New York) and Nancy Johnson (Republican, Connecticut), is urging disease associations to encourage 'grass-roots' support among its members. Although the administration is likely to veto the bill if it is passed by Congress, Waxman aide Tim Westmoreland says, "he [Bush] should at least have to face the Parkinson patients and the diabetics and tell them that he's vetoing it". The proposed legislation promises a heated debate. **Diane Gershon**

scientists to flock to Japan to take up the various new postdoctoral fellowships that have been put on offer.

Now MITI has jumped on the bandwagon. In June the ministry said in its "Ten-year vision for the 1990s" that there is a need to establish a basic research centre to promote international research exchange and collaboration among industry, government and academic institutions. This was quickly followed by a proposal from MITI's Agency of Industrial Science and Technology (AIST) to convert some of the agency's institutes in Tsukuba into a centre of excellence. Last week the fad really took off when an advisory panel of academics and industrialists, the International High-Tech Technology Cooperation Committee, recommended to another section of the ministry, the Machinery and Information Industries Bureau, that Japan should establish such centres of excellence to try to redress the gross imbalance in the flow of researchers between Japan and the western world.

Katsuhiko Umehara of AIST says it is only a "very vague and abstract idea" at this stage but MITI will try "to harness the vitality of the private sector" (a Japanese expression meaning 'ask very politely for money'), perhaps by establishing a private foundation. The foundation would fund a new multidisciplinary research and development centre in Tsukuba. The institute would be open to foreign scientists and might even be headed by one. But Umehara says a foreign director is not an essential ingredient.

David Swinbanks

HUBBLE SPACE TELESCOPE

A discovery and new puzzles

Washington

INVESTIGATORS searching for the cause of the spherical aberration that afflicts the Hubble Space Telescope (HST) have pinned the blame on what had always been the prime suspect. Measurements made last week revealed that one of the elements of the reflective null corrector, an optical device used to assess the shape of the primary mirror during polishing, was misplaced by a millimeter. But this discovery still does not dispel the mystery of the HST: so gross a flaw in the null corrector is as baffling as the error in the primary mirror itself. And to add to the frustration, it has been found that a test of the primary mirror conducted with a second null corrector clearly showed the presence of aberration — but the evidence was discounted because the second corrector was thought to be of lower quality.

Null correctors are needed in the manufacture of astronomical mirrors because of the departure of the mirror profiles from spherical form. An exactly spherical surface has the property that light emitted from a point source at its centre of curvature will be reflected back along radii to the same spot; a failure to achieve focus in this way indicates that the mirror is not perfectly spherical. To test the shape of a hyperboloidal mirror such as HST's primary, a variation on this technique is used. A null corrector is interposed between the mirror and a light source in such a way that light returns to a single focus; the corrector compensates for the asphericity of the mirror. But if the null corrector is inaccurately designed or made, or if it is put in the wrong place with respect to the mirror surface, the mirror's surface will be faulty.

In the manufacture of the HST's primary, two null correctors were used at different stages. The supposedly less accurate one, a 'refractive' null corrector whose optical elements were lenses, was used to check that the shape of the mirror was approximately correct before final polishing began. The reflective null corrector, made of small mirrors rather than lenses, was then used in conjunction with a computer-controlled polishing apparatus to bring the mirror into its final form. Although both null correctors were made to high precision, optical engineers at Perkin-Elmer, the company that made the mirror, believed that reflecting rather than refracting optical elements should be more accurate, and thus that tests conducted with the reflective corrector were more trustworthy.

According to the announcement last week from the National Aeronautics and Space Administration (NASA), the design of the reflective null corrector was as it should have been, but somehow it was put

together wrong. Roger Angel, a mirror-builder at the University of Arizona and a member of the HST board of investigation, says that there was no obvious carelessness at Perkin-Elmer: spacing rods between the components of the null corrector were made and verified to micron accuracy — but they somehow turned out to be off by a millimetre. Angel suggests that although an enormous amount of care was invested in following predetermined manufacturing and testing procedures for all parts of the HST's optical system, "common sense was lost". He points out that the error in the null corrector could have been detected, had anyone thought to try, with a standard plastic ruler.

An equally alarming discovery by the HST investigators was that the refractive null corrector had been used one last time to measure the overall radius of curvature of the mirror after polishing and figuring had been completed. The results of this test, found among the documents impounded by the board of investigation

(see *Nature* 346, 207; 1990), clearly revealed the spherical aberration. Apparently, however, confidence in the reflective null corrector was so great that this finding did not set off any alarm bells.

While Angel and his colleagues continue their work, trying now to discover how a millimetre error in the null corrector occurred and went undetected, other optical experts, including Marjorie Meinel of the Jet Propulsion Laboratory in California, are advising NASA on how best to correct the HST's aberration.

Meinel says that although knowledge of the flawed null corrector will help to pin down the exact form of the aberration and thus to design a corrective lens for the Wide Field and Planetary Camera (see *Nature* 346, 3; 1990), the most accurate measure of the aberration will come from direct imaging tests still being conducted by NASA. One possibility, according to Meinel, is to design corrective elements for the camera and other instruments which include some degree of adjustability, so that final precise error compensation can be done on board HST.

David Lindley

OCEAN RESEARCH

Full fathom five my camera lies

Washington

DESPITE accidentally losing their \$250,000 camera platform more than a mile underwater, deep-sea researchers in Oregon were putting on a brave (if red) face last week. The chance arrival of a deep-diving submarine may mean the rescue of the expensive package, and the resumption of their exploration of volcanic fissures on the ocean floor.

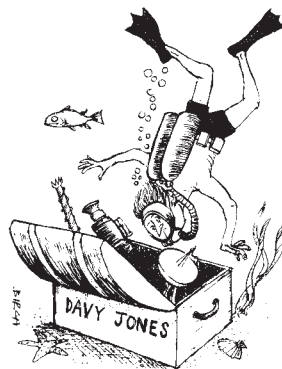
The platform, containing a special camera and a series of related mapping instruments, now sits on the ocean floor 200

heavy weight connected to a number of glass floats and a very strong cable) in the area, to aid in the rescue.

Alvin, Woods Hole Oceanographic Institution's deep-diving submersible, will attempt an unusual rescue mission of the package this week. During the dive, its three-person crew will attempt to find the instrument by searching among the beacons. If they find it, they will try to attach it to the mooring platform and then release the weight. The floats should bring the cable to the surface, where a ship-borne winch can raise the entire 5,000-pound package.

Alvin's rescue attempt will be its second in a week. Last weekend, it was going to try to recover a side-scan radar instrument that a private company lost two months ago. About ten such scientific, mapping or deep-ocean drilling instruments are lost each year, says Barrie Walden, manager of submersible engineering and operations at Woods Hole. But *Alvin*, America's chief non-military submersible, is rarely available to attempt a rescue; it is called on only about once every two years. *Alvin* happens to have been conducting biological and geochemical observations of the same volcanic plume area where the two instruments were lost. "Most of the time when something like this happens, you're out of luck", says Embley.

That *Alvin* happens to have been scheduled for the area anyway, he says with some understatement, is a "fortuitous circumstance." Christopher Anderson



miles off the coast of Oregon, thanks to a defective cable that parted during a routine mapping process. Robert Embley, a geophysicist at the National Oceanic and Atmospheric Administration who is chief scientist for the project, says that his team thinks they have found the platform. They dropped a network of beacons and a special mooring platform (which consists of a

Magellan arrives; all systems go

Washington

AFTER a string of messy failures, the National Aeronautics and Space Administration (NASA) got a rare dose of good news last week when the Magellan radar-mapping probe flawlessly entered orbit around Venus. After two weeks of sensor testing, the craft will begin a 243-day mapping mission that will record Venus's hidden surface in near-photographic quality.

The \$744-million, 3.7-ton probe was launched last May from the space shuttle Atlantis. Last week, after an 83-second braking manoeuvre, it entered an elliptical polar orbit that will allow it to cover 70–90 per cent of Venus' surface. First data are expected to be released on 27 August. Magellan is the fourth craft to orbit Venus. A US Pioneer and two Soviet probes have already mapped much of the planet at somewhat lower resolution. But Magellan, with a high-performance radar-imaging system built by Hughes Aircraft Company, will provide surface information more detailed than currently exists for Earth's ocean floor (see *Nature* 346, 525; 9 August 1990).

Christopher Anderson

CZECHOSLOVAK ENVIRONMENT

Clean-up begins

Prague

CZECHOSLOVAKIA underlined its commitment to cleaning up the environment earlier this month when it created a Federal Committee for the Environment, the first such organization in its history. The government gave sweeping powers to Environment Minister Josef Vavrousek, who will try to improve the situation drastically by the end of the 1990s.

At the same time, the government recited a litany of bad news, the worst of which was that Czechoslovakia would probably not be able to keep its promise to reduce sulphur dioxide emissions. As signatories to the 1979 Geneva Clean Air Convention, Czechoslovakia and 20 other European countries promised a 30 per cent reduction in 1980 sulphur dioxide emission levels by 1993. And Czechoslovaks have an expected life span five to seven years shorter than people in the industrial West while seven per cent of national income is lost through environmental damage.

The environment continues to be the highest priority with the Czechoslovak population, even above money and jobs.

But things may change when the state intervenes to shut down heavily polluting factories or reduces the availability of goods. In the June elections the Green Party did not receive the five per cent of votes that would have guaranteed it a seat in parliament.

Veronika Maxová

Space policy goes green

Washington & London

CONCERN over climate change and environmental degradation is driving a fundamental shift in the rules of international space collaboration, according to officials now negotiating a new policy on scientific data exchanges. European Space Agency (ESA) partners say the requirement that projects should at least partially pay for themselves — a position most strongly argued by Britain and France — is likely to be abandoned for missions of environmental importance, such as the \$40,000-million Earth Observing System (EOS).

The decision appears to remove the last obstacle to full international participation in EOS, a series of large climate-monitoring satellites that will begin operation later this decade. Until recently, EOS partners had been deadlocked over the questions of who should get the data and at what cost. But officials now say that a resolution passed at last month's G7 summit meeting in Houston has paved the way for agreement on free access to EOS data, at no more than minimal reproduction costs, for all qualified researchers.

Agreement by EOS partners to a free-access policy for the project's data will be a victory for the United States and Japan, which have argued that all researchers who agree to publish their results in the open literature should get EOS data free. Although ESA policy is still under negotiation, the agency's standard position has been to allow only participating researchers to get free data. Other scientists and commercial entities traditionally pay for the data, in part to allow ESA and its partners to recoup costs. Even within ESA, however, the issue is debated.

Britain is one of the strongest advocates of commercial space. "What we mean is that we should be in space for a purpose, not just to be in space", says one official at the British National Space Centre (BNSC). That purpose has historically been to make money, a position that has put Britain at odds with some of its ESA partners.

But in recent months, in line with the Houston summit resolutions, commercial concerns have been eclipsed by the concept of a national responsibility for environmental data. "What we're realizing is that when it comes to the environment, the government and the taxpayer should pay for the data," says the BNSC official. That Britain, the country that has fought hardest for a return on its space investment, is considering making an exception for environmental data is seen as an important step towards a uniform ESA data policy of open access.

ESA partners are currently negotiating a data policy for ERS-1, the first satellite

of the agency's Earth Remote Sensing (ERS) programme. The satellite, which will be launched next year, will probably be operated according to a compromise policy in which non-participating researchers will be invited to submit research proposals. Approval by ESA will automatically bring free access to data. Other researchers and commercial entities will have to pay an as yet undetermined access fee for the ERS-1 data.

Guy du Chossois, ERS mission manager in ESA's Paris headquarters, says that the ERS policy reflects a general trend away from a strict commercial approach to something more flexible. Although much of the shift is due to a growing sense of environmental responsibility, the fact that satellite data have rarely been profitable has not escaped ESA's attention. Last year, the US Landsat programme — the largest commercial Earth observation effort — was saved from cancellation only after the White House decided to bail it out with federal funds. And a US experiment in commercializing data from a space-borne wide-field camera was recently ended after it was revealed that total receipts had amounted to less than a thousand dollars. "Everybody knows that you can't recoup your costs", du Chossois says.

Current US policy is to give data freely to qualified researchers in any country that agrees openly to share its Earth-science data and participate in international environmental research programmes. EOS, which will consist of 15 large platforms (six from the United States, six from ESA and three from Japan), is designed to make simultaneous observations with a number of different instruments. To allow scientists to combine data from several instrument, the National Aeronautics and Space Administration (NASA) has decided for the first time to abandon its policy of allowing principal investigators to have a period of exclusive use of the data.

Researchers stress that a non-discriminatory policy on data access is doubly important for an environmental initiative that spans the globe. Although Brazil, for example, is not a partner in the EOS programme, data from the Brazilian rain forests are a crucial part of the climate change equation. Forcing Brazilian scientists to pay for data would "smack of colonialism", and could result in Brazil charging for its ground-based data, says Robert Gurney, an EOS principal investigator at the University of Reading. And influencing environmental policies in the developing nations is far easier when their scientists are participating equally in global change research, he points out.

Christopher Anderson & Peter Aldhouse

GENETIC ENGINEERING

Greens losing gene battle

Munich

OPONENTS of genetic engineering in West Germany are in retreat following a recent licensing decision and the passage of a long-awaited new law prescribing the conditions under which companies may use genetic-engineering techniques.

Last week, the local government in Cologne approved the construction of a production plant at the company Grünenthal AG in Aachen that will use genetically-engineered bacteria to produce the anti-clotting drug Saruplase.

And in Frankfurt, the passage of the so-called 'gene law' led to a swift retreat of

local activists who had appealed to the courts to prevent the operation of a nearly completed pilot plant at Hoechst AG that will produce human insulin from *Escherichia coli* bacteria.

The recent developments give hope to pharmaceutical producers that West Germany will no longer be a black hole for industrial gene manipulation. Despite the good safety record of industrial genetic engineering in other countries, West German officials have permitted one company only (Boehringer Ingelheim) to use genetically engineered bacteria to produce drugs.

GLOBAL CHANGE

New programme launched

San Francisco

IN an attempt to apply interdisciplinary methods to the study of global environmental concerns, a new programme known as the National Institute for Global Environmental Change (NIGEC) was launched this month by the University of California and the US Department of Energy (DOE). NIGEC, which has a \$6 million DOE start-up grant, is a nationwide network of academic research on global environmental issues. The national centre, at the University of California at Davis, will coordinate projects at four regional research centres around the country, in the hope of clarifying the science behind complex problems such as the greenhouse effect, ozone depletion, and general issues of energy use and pollution.

NIGEC will not address policy issues, but scientists see the DOE funding as a sign of increasing government interest in environmental issues. US researchers tend to believe that Western European countries and Japan are the nations "on the cutting edge of making enlightened recommendations" on how to deal with global environmental changes, according to Joseph Knox, acting director of the NIGEC. But he is encouraged by recent US efforts. "The government has been talking about being interested in this area for a long time. It's gratifying to see some actual progress," said F. Sherwood Rowland, a co-director of NIGEC and a professor of chemistry at the University of California, Irvine.

Although some universities have had environmental programmes running for several years, researchers say that the past year has brought an increase in the number of groups studying environmental change on a planetary scale, as well as a tendency to combine a broad mixture of disciplines.

Stanford University in California, for

example, is developing a new undergraduate course, aimed at understanding the Earth as an interactive system; it will mix studies in Earth sciences, biological sciences, economics, policy analysis and environmental engineering. Some classes for the programme, called Earth Systems, are thought likely to begin during the next academic year.

Administrators report bureaucratic obstacles to the creation of programmes that require co-ordination between several academic departments or between different institutions, but Alan Miller, director of the year-old Center for Global Change at the University of Maryland, predicts that increasing demand will push many more universities into beginning such studies with a global theme.

The NIGEC will combine basic scientific research in biological, Earth and atmospheric sciences with studies of such issues as energy policies and fuel consumption. The regional centres, located at Harvard University, Indiana University, Tulane University in New Orleans and the University of California, are designed to foster interdisciplinary research within and among universities located in different parts of the country. As well as attacking global issues, each center will concentrate some of its efforts on understanding the causes and effects of environmental change in its particular geographic and climatic region, said Knox.

Harvard's studies, for example, will include a focus on temperate forests, while the University of California centre will include atmospheric chemistry at Irvine and studies of water resources, agriculture and ecosystems at Davis. At present 60 to 70 research programmes are participating, and the NIGEC will solicit additional proposals later this year.

Elizabeth Schaefer

Both companies can rely on a pivotal clause in the new law to protect them from further appeals. The clause states that public hearings will not be required for the licensing of production facilities that rely on genetically manipulated bacteria in the lowest of four risk classes. Both the Grünenthal and Hoechst plants rely on a bacterium in this category, a 'safety strain' of *E. coli* known as K-12.

The positive decision in the Grünenthal case came after a public hearing last autumn, which was required by the old law governing genetic engineering, the Federal Emission Protection Act. The Cologne head of government, Franz-Josef Antwerpes, used the new gene law as a standard to measure the application. He declared that Grünenthal would go even further than the law requires to protect the local population and its own employees from potential hazards.

Unless there is another appeal, which now seems unlikely, Grünenthal expects to complete construction of its Aachen plant in 1992.

Hoechst had nearly completed its \$60 million pilot plant in 1987, when it was granted a licence to produce human insulin there. But the licence was held up in the courts until this year by the Hoechster Schnüffler und Maagucker, a local opposition group.

Last November, a court ruled that the plant could not be used until there was a legal basis for regulating the use of genetic engineering (see *Nature* 342, 218; 1989). After passage of the new law, opponents declared the suit null and void. Once Hoechst has received official approval from the court, it will complete the pilot plant and apply early next year to build a full-scale insulin plant in Frankfurt.

In cases where other organisms are used to produce drugs, the outcome may not be so clear-cut. Another company, Behringwerke AG of Marburg, is waiting with bated breath for its local government to decide if it may use mouse cells in culture to produce the anti-anaemia medication erythropoietin, also known as EPO. Spokesman Wolfgang Faust said a ruling is expected in September.

Although they are not happy about the recent turn of events, opponents of genetic engineering, such as Green Party parliamentarian Katrin Grüber in North Rhine-Westphalia (where Grünenthal is located) are not ready to admit defeat. "We don't believe that the public ever had a chance to say if it wants medications produced by these methods. We will continue our work by doing public relations and distributing flyers, so that patients will at least know what they are taking." But Grüber admits that it will be impossible to overturn or revise the genetic engineering law without the support of a larger party than the Greens. And that seems unlikely.

Steven Dickman

Where now for taxonomy?

H.T. Clifford, R.W. Rogers and M.E. Dettmann

With the increasing financial squeeze faced by taxonomists, the time may now have come to dispense with massive herbarium collections. Indeed, the clear-out might lead to a better quality of taxonomic research.

It is paradoxical that taxonomy should underpin biology and yet be so poorly supported financially. Accurate identification of taxa is of prime importance to most biological research, and the nomenclature which enables taxa to be discussed is based on type specimens. Accordingly, types must both be carefully preserved and made available for examination by taxonomists. During the past 200 years many thousands of types have accumulated, on top of which herbaria and museums have to make room for a great many botanical specimens used in revisional studies or lodged as vouchers for ecological and other studies.

The announcement that financial stringencies have forced the Natural History Museum in London to dispense with about 100 posts, including 15 per cent of its research staff, provokes several questions, some of which have been discussed in *Nature* over the past few weeks. But one of them concerns botanists. If a world centre for taxonomic studies located in a comparatively wealthy European country is reducing its financial support of taxonomy, other centres are likely to be faced with similar reductions. At this university, we have already been forced to review our priorities on the resources devoted to our small herbarium, causing us to consider the future of taxonomic collections.

As botanical taxonomists we recognize that, for example, much of value has been written about Australian ecology on the basis of an incompletely known flora. Indeed, identifications restricted to a level as high as super-order convey a great deal of the ecological information carried in identifications to the species level. Against that background, do we need to retain massive herbarium collections?

Such collections are immense and still growing. They require large buildings and staff for their curation; there are continu-

ing maintenance costs. For well-known floras, such as those of Europe, can the retention of large numbers of herbarium sheets be justified? What would be lost if label data on all sheets was recorded, a careful selection of sheets kept and the rest pulped? The deposition of voucher specimens to document ecological and other studies is of limited value, for they are rarely studied. Similar procedures could probably be followed for all collections from the rest of the world which have been the subject of revisional studies in the past 30 years. The result would be a huge saving in costs of buildings and their servicing, and in curatorial effort, releasing the diminishing number of staff avail-

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Room for doubt? — a wing of the herbarium at the Royal Botanic Gardens, Kew, London, UK.

able to continue with taxonomic research proper rather than curation.

Few other fields of research require that workers retain raw materials after observations have been recorded. Chemical laboratories do not spend valuable funds housing and caring for millions of specimens of chemicals, a published report being considered adequate. Biological specimens may be different, but they differ only in degree, not nature (except, perhaps, for types). New specimens may be collected for new treatments. If no new material exists (because a taxon is extinct), it may be argued that a taxonomic treatment is of little merit. Palaeobotanical material is, of course, a case apart, and such collections must be retained.

The case for destroying most specimens used in revisional studies or lodged as ecological vouchers is strong, for information about them is available in printed form. Lists of locations could be kept in herbaria and museums, but such accessory publications as are now available are rarely consulted. Should a later revision or ecological survey be required, a selection of materials would still be accessible. Herbarium material is, in any case, no longer accepted as a sufficient basis for taxonomic studies; botanists undertaking revisional studies must try to secure living material from field populations to supplement, if not substitute for, dried collections.

A case can even be made for moving to

Royal Botanic Gardens

an entirely literature-based taxonomic system, in which type specimens are no longer required, but a system of nomenclatural priority is maintained. If taxa are to be subdivided or combined, the decision is made solely on the basis of published descriptions. Such a change would be drastic, but meanwhile we should be more certain about the accessibility of type specimens and the care which they receive if money did not have to be spent on the curation of

thousands of non-type specimens.

If taxonomic botany is to continue, it must do so on a rational economic basis. It is not rational to house tens of millions of herbarium sheets, to continue accumulating them at great price and then adding to the cost by laying each on a large sheet of acid-free card and placing it in a folder of acid-free paper. Far better that the truly valuable specimens are retained and preserved into the future. The measure of a herbarium should cease to be the size of its collection and instead become the quality of its research. □

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East German Academy

SIR—*Nature* has given considerable space to discussions of the problems of integration of East German (GDR) scientists and institutions. Every sensible scientist will agree that this integrated German science system should be based on the existing research and research-funding system of the Federal Republic (FRG), but it is strange that *Nature's* correspondent and the author of a recent leading article (*Nature* 345, 371–372; 1990) refer to the Academy of Sciences of the GDR as if it were a community comparable, in its composition and procedures, to the academies and research organizations of the West. Particularly strange is *Nature's* advice that West German scientists, specifically at the Max-Planck-Gesellschaft, should have “of course, to accept that the East Germany Academy of Sciences should be a titularly equal partner”.

This view of the GDR Academy, however, is shockingly naive, and overlooks some of the ugly facts of life of science in East Germany. Among the members of the grossly overstaffed GDR Academy and the universities one finds, besides many excellent scientists, a fair number who have reached their privileged and tenured positions not because of outstanding scientific achievements but because they were selected and promoted for political reasons, perhaps because they came from families of the Communist party nobility.

A more serious problem is that it has not yet been possible to identify the many East Germany scientists who have served as an internal Secret Service Cadre (“*Sicherheitskader*”) directed by, and reporting to, the GDR version of the Securitate, the *Staatssicherheitsdienst* (“*Stasi*”), spying on their colleagues and on Western visitors. These people were eager to report information indicative of a forthcoming defection of a colleague, and unofficial contacts with Western scientists and non-scientists (“*Westkontakte*”). As in several other Eastern bloc countries, many of the privileged travel cadre (“*Reisekader*”) returning from visits or congresses to the West would report information that they or the *Stasi* found interesting, including details of the private lives of their Western colleagues.

GDR scientists also helped the *Stasi* to identify Western scientists attending research conferences or visiting research laboratories in the DDR who were then placed under observation, with bugged hotel rooms, video cameras and the like. At a dinner during a cell biology meeting near Weimar some years ago, a colleague from a GDR university told me that the same afternoon the *Sicherheitskader* of this meeting, with prominent colleagues of the academy, had decided that I was to

be on the list of those to be secretly observed and, he continued with grim humour, I should take it as an honour.

Academy members were also among the strongest supporters and executors of the suppressive and inhuman science politics of the Party, SED (now shrunk, outwardly metamorphosed and re-named PDS), which excluded for political reasons many gifted and dedicated high school pupils, university students and young scientists from research careers and from international communication. Moreover, some members of the academy were among that most anxious to boycott certain congresses in the West and to vote against GDR membership of international federations, even when most other Socialist countries had already become members (as in the case of the European Cell Biology Organization).

GDR scientists and physicians have also approved or even contributed to secret and inhuman medical manipulations of human beings. These ranged from the spiriting away of some ‘difficult’ and unwanted people to psychiatric ‘sanatoria’ to systematic doping of a whole generation of male and female athletes with neuropeptides and androgenic steroids (in some cases enforced with pressure from the *Stasi*) at a secret government research institute near Kreischa in Saxony, a crime against medical ethics now openly admitted by the new sports leaders of the GDR and some of the professors involved.

Since the ‘change’ in November 1989, only a few of the academy members responsible for these political actions have been removed from office (not from membership), and the influence of the ‘old boy network’ is still felt — the intellectual chameleons have simply adapted from the old to the new. It is nice of *Nature* to be so concerned about the future continuing support of the old science establishment of the GDR, notably the academy, but who cares about its victims? I hear the chorus calling “money”, but who has heard the sentence “I am sorry”? Who has heard a word of apology to those students, scientists and physicians who suffered during the decades of the SED regime? Where are the signs of a will towards reparations and atonement, the invitations of the ‘forgotten generation’ of nonconformist scientists to come to the academy laboratories for a fair second chance?

Unfortunately, recent action in GDR research institutes and hospitals suggests that the leading scientists and physicians have not changed but only dissembled. Members were invited to ‘retouch’ and rephrase their CVs and personal files in the light of the prevailing tone, and doctoral theses with comprising contents ‘dis-

appeared’. This nationwide faking and covering up, however, shows only that the old spirit of opportunistic lies is still there, and to quote Berthold Brecht: “*Der Schoss ist fruchtbar noch, aus dem das kroch*” (“The womb from which it crawled is still fertile.”).

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Tomorrow's world

SIR—Christopher J. Lote (*Nature* 346; 10; 1990) suggests that just about all scientists are atheists and that this hypothesis should be proved and published. He seems to think this would result in popular rejection of religion rather than rejection of science.

More seriously, he seems to think near-unanimity among scientists in being atheists supports the notion of atheism. Alas, humans have a habit of believing in what they are doing. I am a physician, and my discipline takes as an assumption that “the human body is there to be tampered with”. I may have a difference of opinion with most my fellows as to when life “really” begins and “really” ends. A palace guard who took the job just because it was a meal ticket may in time be loyal to the king.

When science was called “natural philosophy”, the great Western religions, Christianity and Islam, took as an assumption the impending fundamental alteration of the Universe. Even Teutonic paganism conceded a twilight of the gods. Greek paganism, which accepted a beginning but no impending end of the Universe, had fallen out of favour. Natural philosophy took as an assumption, “What if what you see is what you get? What if tomorrow is going to be about like today?” The answer, elegantly recorded in your publication, does not go one step toward proving that tomorrow will be like today. Nor does the general agreement of those who work under the assumption support it. Lote should know this.

Actually there is a little theorem in general relativity, the smell of which I feel sure led to Einstein’s “blunder”, that would predict an unstable Universe and an after-death hell quite horrible enough for any Calvinist. I cannot prove the theorem. (Physicians don’t often do that kind of work.) But I can describe it for you. And based on it, I can give a cosmology that can at least be disproved by the proper astronomical measurements.

Certainly religions vary. But many of them are attempts by this tribe or that to come to grips with the question, “What if tomorrow is different?”

M. L. HERBERT

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Database activity in Japan

SIR—I found the recent article "New computer link for Japan" (*Nature* 344, 92; 1990) misleading in its comparisons between Japanese and Western database activities.

First, as the article states, the manpower at the DNA Database of Japan (DDBJ) is certainly smaller than that at the European Molecular Biology Laboratory (EMBL) or GenBank, which are working with different entry-formats. In contrast, DDBJ did not invent its own third format; it adopted the GenBank format. This is an important contribution to worldwide database activities, as a third format would have introduced unnecessary problems among users.

Second, the lack of IBM-compatible computers in Japan does not cause serious problems in building databanks. In JIPID, we are accepting sequence files created on IBM-incompatible NEC personal computers. The problem of converting the software 'authorin' can only be a temporary situation. The question is not what is the standard but whether one is available.

Third, the article says that the DDBJ does not have the capability to cope with the massive amount of data expected to arise from the Human Genome project. This is probably true. However, it is also true of GenBank and EMBL, in terms of today's facilities and technology. An underlying assumption of the human genome project is that it will be able to take advantage of — or even itself to stimulate or generate — the technical advances that it will require. It is therefore misleading to suggest that only the DDBJ faces such problems.

Readers may derive from the article negative impressions of the contributions of Japanese groups to worldwide database activities. In the case of amino-acid sequences of proteins, JIPID (Japan) has formed an international collaboration with NBRF (USA) and MIPS (FRG) called PIR-International, which has adopted a common data-entry format. Journal scanning is shared among the three databases according to their geographical areas; a similar collaboration exists in the field of nucleic acid sequences among GenBank, EMBL and DDBJ. For example, JIPID collects protein sequences published mainly in Asia and Oceania. The number of journals scanned in JIPID is 74 out of a total of 162 journals covered by PIR-International. Sequence data entered in JIPID amount to 20 per cent of PIR-International. Besides this protein database, JIPID works on developments of specific genome databases, such as bacteriophage T4, *Escherichia coli*

and rice. Variant and enzyme databases are also being developed for molecular biology and biotechnology.

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Overseas voters

SIR—Many recent leading articles in *Nature* have drawn attention to the shortage of funding for science in Britain, particularly in the university sector, and concern has been expressed at the number of British scientists leaving the country to work in conditions more conducive to their scientific and career goals. British scientists living abroad may therefore be interested to know that anyone who has left the country since 11 October 1970 is now entitled to vote in UK and European parliamentary elections. It is no longer necessary to declare an intention to return to the United Kingdom.

Overseas electors can vote in the constituency in which they or their family were registered before leaving the United Kingdom, provided they complete an application form (available from their nearest diplomatic or consular post) before 10 October 1990.

As well as having a say in the democratic process at election times, British scientists abroad will thus have a Member of Parliament whom they can lobby on any relevant matter. I hope that all who are concerned for the future of science in Britain will welcome this chance to vote for the political party most likely to provide appropriate funding for basic research in the universities and elsewhere. Such a change might even attract back some of the talent currently lost to the nation.

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Genome research

SIR—Your Australian correspondent, Tania Ewing, in a recent News article on "Preserving the present" (*Nature* 345, 465; 1990), has misrepresented my views and the attitude of the Australian government to the collection of genetic resources and strategic genome research.

The Gene Library is being established to preserve and analyse DNA from a broad spectrum of species from the Australasian region, with particular focus on those that are rare, threatened or have unusual evolutionary or biochemical features. While we have not as yet received direct funding from government sources, we have not voiced complaints,

and there is increasing interest from this sector. Indeed, collection of samples for the library has recently been made a condition of permits to take wildlife issued by the Queensland National Parks and Wildlife Service. We have also received an enthusiastic response from museums, herbaria, other wildlife agencies and research laboratories around Australia, through which we have access to their large existing collection of biological material.

The quotation ascribed to me that the Australian government "can't see the benefit of . . . sequencing key genomes" and the imputation of complaint are totally inaccurate. The Australian government, via the Department of Industry, Technology and Commerce (DITAC) and other agencies, is very much interested in and supportive of strategic genome research. Indeed, DITAC has established an *ad hoc* committee, of which I am currently a member, to promote and coordinate such research with, in all likelihood, additional directed funding. The suggestion that the Australian government is unaware of or unresponsive to the importance of these areas is the opposite of the true situation.

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EB research grants

SIR—Epidermolysis bullosa (EB) is a relatively rare, but frequently severe and sometimes lethal, condition which is inherited in various forms and causes blistering to the skin and internal body linings together with other complications. DEBRA is the national charity working to fund research into EB and support children and adults with the condition.

Research work is in progress at a number of centres into EB and, so far, an involvement of type VII collagen and a proteoglycan has been shown. DEBRA wishes to increase the volume of good quality research which it funds and welcomes research grant applications from workers who can bring an innovative approach to the study.

Those interested should contact me at the address below (telephone: 0344 771961), for a list of publications on the subject and a grant application form. Grant applications will be assessed by a specialist medical and scientific panel and will be competitive.

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(Director)

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Weather and the Earth's rotation

A model calculation has shown how the speed of the Earth's rotation differs between the northern summer and the northern winter because of the seasonal variation of the force between the atmosphere and the spinning Earth.

EVERYBODY KNOWS that the speed of the Earth's rotation is not as strictly constant as those who sought to use the Earth as an accurate timekeeper were fond of supposing, but the causes of the measured fluctuations in the length of the day, or LOD, remain to be unravelled. That there should be a secular trend to less rapid rotation on account of tidal interactions within the Earth–Sun–Moon system is of course well understood, and is the chief reason for the now routine annual retrospective corrections, of the order of a few milliseconds, of the length of previous years. There are also regular seasonal variations of the speed of rotation occasioned by the different responses of the two hemispheres to solar heating.

But the short-term fluctuations of rotation rate are much less well understood. The notion that variations of weather patterns that change the angular momentum of the Earth's atmosphere may be responsible is natural enough, but there has been no compelling demonstration that the effects of weather are sufficient to account for the observed fluctuations of rotation rate. One obvious difficulty is that direct correlation of fluctuations of the LOD with measurements of the angular momentum of the atmosphere would require an unattainable degree of sophistication in meteorological measurement. But G. J. Boer from the Canadian Climatic Centre at Downsview, Ontario, may now have found a way of settling the issue (*Journal of Geophysical Research* **95**, 5511; 1990).

The inevitable starting point is the elementary consideration that the angular momentum of the Earth must be constant at least within the framework of the small secular trend to increasing LOD. This in turn implies that the sum of the angular momentum of the solid Earth and the angular momenta of its movable components must be constant. But the movable components are the atmosphere and the oceans. The Earth's molten core is also capable of a fluctuating angular momentum, and indeed does influence the LOD (see J. Lahr, *Nature* **345**, 476; 1990) but on much longer timescales than are considered here.

Boer's trick is to use the Canadian Climatic Centre's model of the general atmospheric circulation to calculate the transfer of angular momentum between the different components of the spinning

Earth. Of necessity, this approach cannot yield a direct correlation between particular weather patterns and particular excursions of the Earth's rotation rate, but Boer is at least able to demonstrate that transfers of angular momentum between the atmosphere and the solid Earth can indeed account for LOD fluctuations of the magnitude observed. Boer acknowledges that his work is by no means the first of its kind and in particular refers to a study five years ago by R. Swinbank at the UK Meteorological Office.

The interaction between the atmosphere and the solid Earth consists of the stresses caused at the interface between them by relative movement of the atmosphere otherwise known as wind. For practical purposes, three parts of the Earth's surface require separate treatment.

First, mountain ridges and other topographical variations of surface altitude present obstacles to wind; the stresses they cause are most simply determined by the differences of pressure across them. But there are also frictional forces at topographically unremarkable solid surfaces, while at oceanic parts of the interface there may in principle be a literal transfer of angular momentum from the atmosphere to the oceans rather than a stress. Plainly it is a matter of some importance to estimate the relative magnitude of these different contributions to the stress on the spinning Earth and thus to the torque which affects its rotation rate. Boer does acknowledge that Swinbank's earlier work had shown mountain ranges to be dominant.

The procedure is straightforward. In the style of atmospheric modellers everywhere, Boer uses a set of experimental measured data of the condition of the atmosphere as initial conditions for his model, discarding predictions during an initial period while the model settles down. In practice, he then allowed his model to run for the equivalent of 20 years and used the stored data produced as a basis for data calculations. As atmospheric models go, the Canadian is reasonably sophisticated. It allows for radiative processes within its ten-level vertical structure, but sea surface temperatures and average cloudiness must be provided empirically. The model does not accommodate phenomena such as the El Niño mechanism, which may nevertheless

be highly relevant for the issue of the Earth's angular momentum.

For outsiders, the sheer size of the quantities involved, however unsurprising, is bound to be striking. In SI units ($\text{kg m}^2 \text{s}^{-1}$ for angular momentum), the angular momentum of the atmosphere appears to fluctuate with the seasons between 15.0 and 25.0 $\times 10^{25} \text{ kg m}^2 \text{s}^{-1}$. The atmosphere in other words is a gigantic flywheel of sufficient size to affect the spinning of the solid Earth over which it lies. As it happens, the angular momentum of the atmosphere as a whole is greatest in the northern spring (March and April) and least in the northern summer (June and July). That there should be such an asymmetry is not surprising. The angular momentum of the atmosphere is necessarily a balance between the easterly winds of the tropics and the westerlies that prevail in mid-latitudes. That these balance differently in the two hemispheres is apparent from Boer's calculations of the angular momenta in the separate hemispheres; that in the Northern Hemisphere falls to zero in the corresponding summer.

The calculations also show the relative importance of the various forces on the spinning Earth which lead to the observed fluctuations of the LOD. Generally speaking, the torque on the spinning Earth transmitted to the oceanic surface is small compared to the forces transmitted through land surfaces, at mountains and elsewhere. Moreover, the oceanic forces are fairly constant from one season to another. Indeed, such variations of the oceanic forces as there are appear most conspicuously at mid-latitudes in the Southern Hemisphere.

But Boer cautiously acknowledges that the calculations of the torque transmitted through the interface between the atmosphere and the spinning Earth are still incomplete. In one particular respect, for example, it appears to be accepted that the sensitivity of the model calculations to the assumptions made about the topography of the Earth is too great for comfort. Even so, to have made such a detailed compilation of the forces that may influence the spinning rate and thus the length of day is a considerable achievement with which timekeepers, not to mention meteorologists, will have much fun.

John Maddox

Undecidable dynamics

Charles H. Bennett

ALLUDING to the effects on twentieth-century physics of quantum mechanics and general relativity, the cosmologist John Wheeler once remarked that the world is simpler and stranger than we can imagine. An analogous dose of strange simplicity was delivered to mathematics in the 1930s by the discoveries of Gödel, Turing and their contemporaries in the theory of proof and computation. Yet there have been surprisingly few applications of these ideas to physics. In particular, the phenomenon of 'undecidability' has not asserted itself in any simple, uncontrived physical setting the way that deterministic chaos, a far weaker property, has. (For a dynamical system to be chaotic means that it exponentially amplifies ignorance of its initial condition; for it to be undecidable means that essential aspects of its long-term behaviour — such as whether a trajectory ever enters a certain region — though determined, are unpredictable even from total knowledge of the initial condition.) But C. Moore

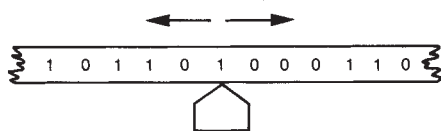


FIG. 1 A universal Turing machine consists of an infinite passive tape and a finite active read/write head. It can be programmed, through the initial state of its tape, to perform any computation.

has now come closer than any to a simple dynamical representation of undecidability.

The theory of computation encompasses any situation in which discrete symbols are manipulated according to a fixed program or algorithm. A distinctive feature of computation is the ability to program an open-ended search for something that may or may not exist, such as a counterexample to Fermat's Last Theorem, which asserts that the equation $x^n + y^n = z^n$ has no solutions in positive integers for $n > 2$. If a counterexample exists, the computation will find it, but if it does not exist, the computation will continue forever without finding it. The lack of any general procedure for deciding *a priori* whether computations terminate, called the 'unsolvability of the halting problem', is the central negative result of computability theory. Not only termination, but any other global property of computations that might be used to signal the outcome of an open-ended search, such as whether a certain bit is ever turned on, is similarly algorithmically undecidable.

The central positive result of computability theory is the existence of 'universal computers', which can be pro-

grammed to simulate any other computer and perform any digital computation. Computational universality manifests itself in the well known ability of real computers (other than specialized ones like those in digital watches) to simulate one another, so that they differ in speed and convenience, but not in the range of computations they can be made to perform. By extension, a discrete or continuous dynamical system is called computationally universal if it can be programmed through its initial conditions to perform any digital computation.

For example, the cellular automaton game Life — in which the state ('alive' or 'dead') of each cell in an infinite array of cells changes according to the states of its neighbours — has been shown to be computationally universal. One can therefore construct an initial condition for it that will implement an arbitrary computation, such as conducting a search for counterexamples to Fermat's Last Theorem and turning on a designated cell when a counterexample is found.

Universality and undecidability are closely related: roughly speaking, if a universal computer could see into the future well enough to solve its own halting problem, it could be programmed to contradict itself, halting only if it foresaw that it would fail to halt. We are thus left with a tantalizing situation. On the one hand, the global map of any computationally universal process — the iterative limit of its operations — is an infinitely valuable microcosm of all iterative processes and all cause-effect relations that can be demonstrated by deductive logic or numerical simulation. On the other, because of the unsolvability of the halting problem, this same global map cannot be computed directly. It can only be approximated as the limit of an iterative process that converges with uncomputable slowness.

Computational universality was originally demonstrated for a very simple type of ideal computer, the Turing machine, which consists of an infinite passive tape, on which data is stored in the form of binary digits 0 and 1, and a finite active head which shifts the tape back and forth manipulating the data one bit at a time (Fig. 1). It is this type of computer which Moore has recently succeeded in embedding in a continuous dynamical system¹. Ideally, as Moore points out, one would like to prove computational universality, and undecidability, for some famous problem in continuum dynamics, like the three-body problem — the calculation of the orbits for three interacting masses. Moore's system, while still simple, is less natural: it corresponds to the motion of

a single classical particle in a three-dimensional box containing a finite number of plane and parabolic mirrors.

Moore's construction generalizes the ability (Fig. 2) of simple chaotic dynamical processes to split a parallel bundle of trajectories in two, deform and reunite the parts, then feed them back into the original bundle in such a way that trajectories are continually pulled apart in one direction (here horizontally) and squeezed together in another (here vertically). Associated with any such process is a certain fractal set of initial conditions shown in the enlarged cross-section of the bundle and called a Cantor set. This set

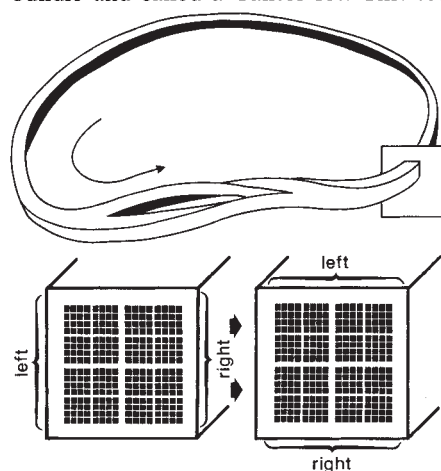


FIG. 2 Typical chaotic dynamics has the effect of splitting a bundle of trajectories into two parts, then deforming and merging them to produce a simple deterministic shuffle of the associated Cantor set visible in the enlarged cross-section. Moore's generalization of this splits the bundle into several dozen parts, then deforms and merges them to produce a more complicated shuffle equivalent to the action of a Turing machine.

has the form of a solid square from which a vertical and a horizontal cut have been removed leaving four smaller squares, each of which is further subdivided in the same way *ad infinitum* until no solid contiguous pieces remain. The dynamics has the effect of performing a rather simple deterministic shuffle of all the infinitely many points in the Cantor set, so that each point in the left half of the Cantor set, after one turn of dynamics, ends up as a corresponding point in the top half, and each point in the right half ends up in the bottom half.

This shuffle can be described more abstractly as a repeated left-shifting of an infinite binary sequence whose left and right sides represent, respectively, a point's vertical and horizontal address within the Cantor set. Left-shifting a sequence without changing it is just a rather trivial Turing-machine computation, and Moore's particle-and-mirror system manages to generalize the dynamics enough to implement a full range of Turing-machine actions needed for universality. Plane mirrors are used to split

and merge trajectory bundles to implement reading and writing, and the parabolic mirrors are used to squeeze and stretch the bundles to implement shifting.

Moore's is the most economical dynamical model of computation as it uses just three dynamical variables (the coordinates of a single particle) to encode the entire logical state of the computation. But, because logically distinct states are packed arbitrarily close together in physical space, Moore's dynamics cannot tolerate any amount of disturbance, however small, by external noise.

A less economical, but otherwise far simpler and more straightforward embedding of computation in dynamics is provided by Fredkin's billiard-ball model², which uses one ideally hard, frictionless billiard ball to represent each bit of data, and performs all necessary logic operations by causing the balls to collide with one another. Like Moore's, this model is reversible: ideally its operation would consume no energy, and reversing the velocities of all the balls (or Moore's one ball) would cause the motion to run backward, exactly undoing the computation.

The differential equations describing a real electronic computer provide another dynamical model at the same level of economy (a few dynamical variables — voltages and charges — per bit). Unlike the preceding models, an electronic computer can recover from small disturbances due to external noise: the ability to do so depends both on having enough dynamical variables to keep logically distinct trajectories physically far apart, and

also on the equations' irreversibility, manifested in a real computer's consumption of electricity and production of heat.

At the opposite extreme from Moore's is a model sketched by Omohundro³, which simulates an arbitrary cellular automaton by a set of space- and time-independent partial differential equations, thereby using infinitely many dynamical variables to encode each bit. Like a real computer, it is irreversible and can recover from small disturbances due to noise; combining it with recent error-correcting cellular automata^{4,5} should yield models also able to recover from the large but rare disturbances characteristic of natural noise sources. Despite their infinitude of dynamical variables, partial differential equations provide the most natural mathematical model for processes occurring in uniform physical space-time, such as fluid flow and wave propagation. So, models of this type hold out the hope that some totally structureless homogeneous medium — an exotic chemical brew, say — might have universal computing abilities, just as simpler brews have the ability to crystallize or to generate spontaneous chemical waves and oscillations. □

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PROTEIN CHEMISTRY

Founding fathers and families

Carl-Ivar Brändén

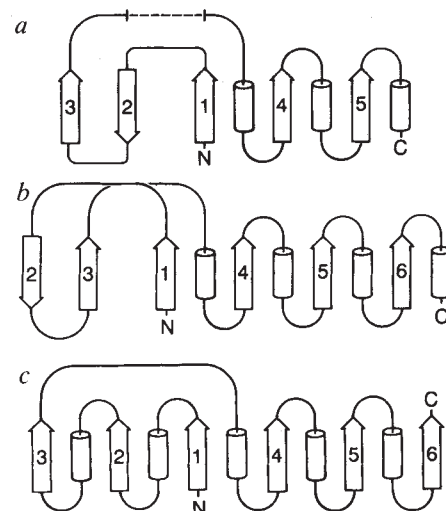
SIMILARITY between the three-dimensional structures of two proteins need not necessarily imply that their functions are similar. But on page 623 of this issue¹, Flaherty *et al.* suggest that major conformational changes play an important part in the function of heat-shock proteins, on the basis of the extensive structural similarity they observe between the ATP-binding domains of the heat-shock protein hsp70 and the enzyme hexokinase, the archetypal example of an enzyme activated by 'induced fit' when a substrate is bound.

Members of the hsp70 family of proteins are ubiquitous and have highly conserved amino-acid sequences². Some members 'escort' other proteins into cell organelles such as the endoplasmic reticulum, the chloroplast and the lysosome. By acting as molecular 'chaperones'³, they prevent their charges from meeting or aggregating with other molecules and ensure that they are translocated in the

unfolded form through membrane pores. Other members of the hsp70 family can bind to and, in the presence of ATP, dissociate protein complexes.

Flaherty *et al.*¹ have worked on one such member, the enzyme that removes clathrin molecules from coated vesicles. This polypeptide of relative molecular mass (M_r) 70,000 hydrolyses ATP to drive the energetically unfavourable release of clathrin. Its amino-acid sequence shows about 50 per cent homology with other members of the hsp70 family, which effectively means that any gross structural information derived from this protein is relevant for all members of the family.

The ATPase activity and the clathrin-binding function of this enzyme reside in two different domains within the polypeptide chain. Although there are numerous examples of these two-domain proteins — the most famous being the *lac* repressor — they are often difficult to crystallize, presumably because of a flexible hinge region



Topologies of the folds of three families of nucleotide binding α/β proteins. Cylinders represent α -helices and arrows β -strands. *a*, The ATPase fold¹ for clathrin-uncoating ATPase; *b*, The G-protein fold that binds GTP and is found in *ras* proteins⁵; *c*, The Rossmann fold that binds NAD in several dehydrogenases⁴.

between the domains. When this is the case, the standard procedure is to attempt to crystallize individual domains and relate their structures to the protein's function. Flaherty *et al.* have determined the X-ray structure of a M_r 44,000 amino-terminal fragment of the uncoating ATPase which retains the ATPase activity of the complete protein but does not bind clathrin. The polypeptide chain of this fragment folds into a bi-lobate structure with a deep crevice between the lobes in which ATP binds. Each lobe has a similar structural core built from five β -strands and three α -helices (*a* in the figure).

Most kinases and dehydrogenases as well as many other proteins have cores built of alternating α -helices and β -strands, so-called α/β structures. These structures and their disposition form the basis of different structural families, depending on the number of β -strands, their orientations and the way they are connected. Thus the NAD-binding domains of several dehydrogenases⁴ have six parallel β -strands in a specific arrangement, the Rossmann fold (*c* in the figure), and GTP-binding proteins⁵ form a family of structures containing five parallel and one antiparallel β -strands (*b* in the figure), the G-protein fold. From the structure of uncoating ATPase¹ and that of the similarly folded actin molecule, determined by Ken Holmes and colleagues and soon to appear in *Nature*⁶, we can now define a third family of such α/β proteins which have a structure that could be called the ATPase fold.

The founding father of this family is the glycolytic enzyme hexokinase, the structure of which was determined more than 10 years ago⁷. The hexokinase polypeptide chain is folded into two lobes, the central core of each having the same

ATPase fold. But despite some structural similarity, there is no overall sequence homology between the two halves of the polypeptide chain in the uncoating ATPase. Flaherty *et al.* demonstrate some sequence similarity between the β -strands of the central core and suggest that the two halves arose by the duplication of an ancestral gene encoding the five-stranded ATPase fold. It is tempting to carry this speculation further and raise the question of whether all proteins with the ATPase fold are related in an evolutionary sense. The same ancestral gene would then have been recruited to encode proteins that could use ATP for such diverse functions as phosphate transfer by hexokinase, chaperone function by hsp70 and muscle action by actin. If this were so, perhaps a similar ATPase fold will be found in other ATP-dependent chaperones, such as⁸ GroE, a member of the hsp60 chaperonin family.

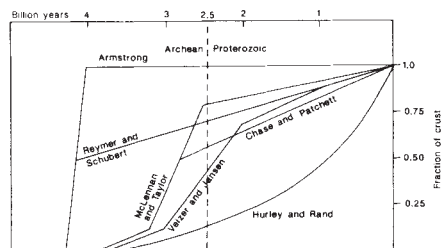
Because the complete structure of the uncoating ATPase remains unknown, it is as yet impossible to say how the energy released by the hydrolysis of ATP is used to liberate clathrin molecules from coated vesicles. Nevertheless, X-ray studies of the related enzyme hexokinase⁹ have provided some clues for comparison by showing that the binding of the substrate glucose induces a large conformational change in the enzyme from an inactive to an active form. On the rather weak basis of the structural similarity with hexokinase, Flaherty *et al.* suggest that the uncoating ATPase may operate by a similar conformational change. They go on to suggest that changes in the ATPase fold are transmitted to the clathrin-binding domain and induce a conformational change in the target substrate so that clathrin is released. This attractive suggestion is only one of many possible routes for the transduction of the energy of ATP hydrolysis to a bound substrate. Further structural studies on complete chaperone molecules with and without bound target substrates, now in progress in several laboratories, are needed to clarify the structural basis for the action of chaperones. □

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Not mere scum of the Earth

Stuart Ross Taylor

THE question of the origin and evolution of the continental crust has long been an interesting geological problem on which considerable progress has been made over the past 30 years, as new isotopic and geochemical information has become available. Opinions have been divided between the proponents of the idea that the crust formed early in the Earth's history, and those who propose that it grew more gradually throughout geological time. These are usually referred to as the no-growth and growth models, respectively. Ross Stevenson and Jon Patchett¹ have now carried out a test that provides new evidence in favour of the growth models.



Various curves proposed for the evolution of the continental crust, ranging from the no-growth model of Armstrong⁵ to various models of growth through geological time^{4,6-10}. Adapted from ref. 1.

The continental crust, typically 40 km thick and covering at present about two-fifths of the surface of the Earth, accounts for only about 0.4 per cent of the Earth's volume. However, it is significant not only in providing a platform on which we can discuss the subject, but also in that it contains perhaps 30 per cent of the total terrestrial budget of the heat-producing, radioactive elements (K, U and Th) and other elements (such as Zr, Ba, Rb and Cs) whose size or valency excludes them from the common minerals in the Earth's mantle. The continental crust, rich in granite and associated rocks, differs fundamentally from the denser, thinner oceanic crust composed predominantly of basaltic lavas.

Early workers supposed that the continents formed as a residual scum on the surface of an originally molten Earth, but this view has not survived the demonstration that large amounts of granite cannot be derived by a single-stage melting process from the mantle (in contrast to the oceanic crust). Rather, the continental crust is the product of several stages, including an episode of massive intracrustal melting that produced the familiar granite and granodiorite which dominate the upper crust.

No similar crusts appear to have formed on the other planets or satellites. Their crusts are mostly basaltic or icy; in the

special case of the refractory Ca-Al-rich Moon, the crust consists of anorthosite. The common and familiar granite is probably unique to the Earth.

Some of the present models for crustal growth are shown in the figure, adapted from Stevenson and Patchett's paper. This figure clearly illustrates the wide difference of opinion between those who propose the formation of an early crust and those who favour gradual growth throughout geological time, by processes still in operation (following lyellian orthodoxy).

One of the principal arguments used by the no-growth, early-crust school has been the 'freeboard' concept. The argument is that continents have always had about the same volume relative to oceans, so that the sea level has always been within a kilometre or so of its present level. Flat-lying Proterozoic sediments provide good evidence extending back for two billion (10⁹) years or so for this effect. Although constraints from the freeboard record are now considered less stringent than originally thought, the argument is basically valid as far as it goes, that is, back into the early Proterozoic, about half-way through the geological record. Before that time, the sedimentary record becomes too fragmentary to make freeboard a real constraint on crustal volume².

Most growth models, as is apparent from the figure, accommodate the freeboard constraint by proposing extensive episodic growth in the late Archaean or early Proterozoic, with only minor additions since that time. There is considerable independent geological evidence supporting this view. The debate thus turns principally on whether there is evidence of extensive continental crust in the older Archaean before about three billion years ago.

What Stevenson and Patchett have now carried out amounts to an independent test of these models, recalling the adage that the process of science depends on the erection of testable hypotheses. If there were large volumes of continental crust present in the early Archaean, about 4 billion years ago, some trace or fingerprint should remain, even if much had been recycled into the mantle.

What would constitute a suitable fingerprint that might survive on the surface of a dynamic planet? Zircon (ZrSiO₄) is a highly durable mineral, common as a trace constituent in granites. It crystallizes from granitic melts when the concentration of the trace element Zr becomes high enough for the mineral to precipitate. Zircon is highly resistant to weathering, being concentrated in quartzites (and in beach sands from which it is mined). It can

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survive through many geological cycles of weathering, erosion and deposition of sediments.

Stevenson and Patchett argue that if early granitic crust existed, then a population of ancient zircons, derived from it by erosion, should have survived and should persist in younger sediments in the geological record. It was a necessary prelude to this test that zircons extracted from such sediments could be dated, and in an earlier important contribution³, Patchett and Tatsumoto developed direct age measurement of zircons by using the radioactive decay of ¹⁷⁶Lu to ¹⁷⁶Hf.

Thus, if the no-growth model is correct, there should be an identifiable population of old zircons, derived from the early crust, present in younger sedimentary rocks. If the growth model is correct, old Archaean zircons should be rare or absent in younger rocks. Stevenson and Patchett have now studied zircons, extracted mainly from quartzites, from several different regions, including the Canadian Shield, and the Wyoming, North Atlantic (Labrador, Greenland, Scotland) and Kaapvaal (Southern Africa) cratons. What they found is that the zircon populations in Archaean sediments are mostly of the same age as that of the terrain in which they are found, and that there is a scarcity or absence of significantly older zircons derived from pre-existing crust. In contrast, younger sediments of Proterozoic age contained a significant population of zircons derived from late Archaean (3.0–2.5 billion-year-old) rocks. This suggests that there was rapid continental growth starting about 3.0 billion years ago, but that before that time, there were only scattered small areas of continental crust.

The basic conclusion is that old continental crust was rare and no more extensive than the few remnants currently exposed. This provides a decisive test in favour of growth models.

Stevenson and Patchett are suitably cautious and address various caveats, both analytical and geological. They analysed zircons mostly from low-grade metamorphic rocks (mainly quartzites) to avoid the problems of overgrowth, which would give spurious young ages. If there was an early extensive basaltic crust, few zircons

would be expected. Most terrestrial planets generate basaltic crusts and there are good reasons for supposing that an early basaltic crust existed on the Earth (Venus may be an analogue). But the debate turns not on the presence or absence of basaltic crust, but on the existence of granitic (sialic) continental crust. Early Archaean continental crust was probably dominated by sodium-rich granites (tonalites) rather than the potassium granites of the present upper continental crust. This is in fact a tenet of the growth model⁴. But even in these rocks, zircons are fairly common, so that the present test of the models is applicable.

Are there other fingerprints that could point to the former existence in the Archaean of massive early continental crust? The depletion of Eu in normalized rare-RNA EDITING

earth-element patterns in sedimentary rocks is typical of post-Archaean crust, and is ubiquitous, occurring for example in deep-sea sediments, in which it arrives as wind-borne dust from the continents. In the Archaean, land vegetation was absent, so that the dust derived from the exposed barren surfaces must have been common and widely distributed. The absence of the Eu depletion fingerprint in the common Archaean greenstone belt sediments similarly argues that the exposed continental crust in the Archaean was probably not much more extensive than is currently exposed. □

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Copy editing — what's the U's?

Elizabeth H. Blackburn

RNA editing involves the addition and deletion of non-coded ribonucleotide residues to and from RNA transcripts, thus converting them into correctly translatable messenger RNAs (mRNAs; reviewed in refs 1,2). The process is dramatically evident in (although not confined to) certain mitochondrial mRNAs of kinetoplastid protozoan parasites such as *Trypanosoma brucei* and *Leishmania tarentolae*, in which 'pre-edited' mRNAs are transcribed from 'cryptogenes', which can be considerably shorter than their counterparts in other mitochondrial genomes. These pre-edited transcripts are corrected mainly by additions but also by removals of uridylyl (U) residues, apparently by using the sequence information separately encoded in one or more short 'guide' RNAs³. Editing can occur in either a precisely defined domain or, in some cases, over the entire transcript. Some new papers⁴⁻⁸ furnish additional tantalizing data about this extraordinary biological process.

RNA editing raises many questions: the identity of the mechanism by which guide RNAs specify the sequence of the edited RNAs; how regions to be edited are recognized as substrates for the editing process; and how RNA editing arose in evolution. Is this baroque procedure important for some aspect of mitochondrial function? Or are cryptogenes an evolutionary precedent to the mirrors of the Hubble Space Telescope — expensive mistakes in the manufacture of transcripts that have to be corrected, at great trouble and expense, by guide RNAs (equivalent to the corrective optics of telescope)?

The mitochondrial DNA of kinetoplastid protozoans is unusual in being comprised of about 25–50 maxicircles — which

encode limited numbers of mitochondrial RNA and protein components and are functionally equivalent to more conventional mitochondrial DNAs — and 10³–10⁴ topologically catenated, smaller minicircles. The recent discovery⁵ that minicircle as well as maxicircle DNAs encode guide RNAs gives minicircles their first (but not necessarily only) recognizable function.

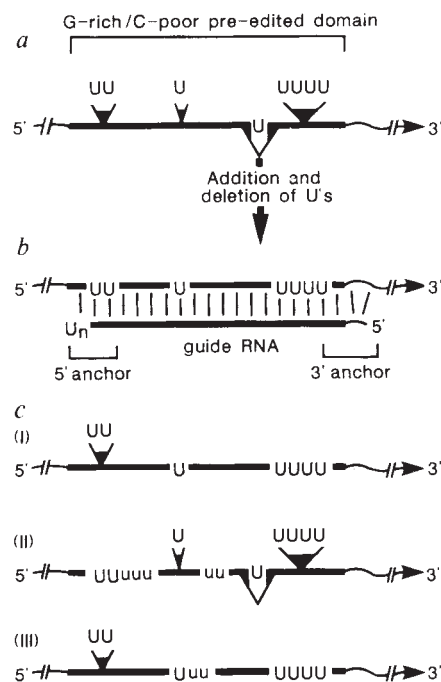
The sequences of the short guide RNAs correspond to patches of the correctly edited mRNA sequence. A model from Simpson's group^{3,8} for RNA editing (see the figure, overleaf) proposes first, pairing of the guide RNA with the pre-edited RNA, the initial pairing being stabilized by anchor sequences at both ends of the guide RNA, including the newly found non-encoded U tract at its 3' end⁶; second, an 'editosome' adds or removes U residues, as specified by the guide RNA sequence, and moves along the paired region in the 3' to 5' direction⁷ (with reference to the mRNA). The editosome is proposed to carry the enzymes necessary for RNA cleavage, addition or deletion of U residues and RNA ligation. An important component of this model is that the sequence of the edited RNA is specified by base-pairing with the guide RNA. Although unusual because it invokes G·U pairs, which are not used in other reactions involving RNA templates, this model is at variance with more complex schemes, in which the secondary structure of pre-edited RNA somehow specifies the positions and numbers of U residues to be added and deleted.

Until an *in vitro* system for RNA editing is developed, or genetic evidence is available, the sequences of guide RNAs remain the strongest single line of evidence

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for their role in RNA editing. But the stage in the process at which they act is still not clear. Although the sequences of some partially edited transcripts suggested that the entire editing process proceeded along the mRNA in a 3' to 5' direction² (c in the figure), others^{4,7} are hard to reconcile with such a model (ii and iii in the figure). Strikingly, these partially edited RNAs have U residues indiscriminately added to or deleted from both correct and incorrect positions only within a precisely defined editing domain⁷. These observations suggest a revised model⁷ including first, recognition of the domain to be edited; second, cycles of indiscriminate additions and deletions of U residues within this domain; and third, 'zipping-up' of the guide RNA(s) with the partially edited mRNA in a 3' to 5' direction, with the cessation of addition and deletions of U residues only when the guide RNA is fully base-paired with the mRNA.

Whether they extend over the entire mRNA or are confined to highly localized segments, nearly all pre-edited mRNA domains have a striking strand bias of G over C nucleotides⁶, although they share



RNA editing of kinetoplast mRNAs. *a*, A pre-edited transcript of a cryptogene. RNA editing adds (Us above) or deletes (U below) uridylyl residues at the positions indicated. *b*, The final correctly edited mRNA (above) whose sequence is specified by a guide RNA (below), whose 5' sequence is complementary to a stretch of up to 16 nucleotides 3' to the pre-edited domain (3' anchor). A non-encoded tail of U residues on the 3' end of the guide RNA forms a base-paired 5' anchor with the 5' end of the pre-edited domain. *c*, *i*, Partially edited intermediates indicating that editing progresses 3' to 5'; *ii* and *iii*, Other partially edited RNAs containing U residues incorporated in wrong positions and numbers within the editing domain.

no other sequence similarities. Could G-richness be a basis for recognition? There may be a precedent in telomerase, the enzyme involved in nuclear chromosomal telomere synthesis. The telomeric DNA strand that serves as a primer substrate for telomerase also has a composition bias of G over C residues, and this G-rich strand is apparently recognized by telomerase on the basis of G-richness, as opposed to sequence specificity⁸. G-rich, C-poor DNA can assume folded structures stabilized by non-Watson-Crick G_{anti}-G_{syn} base pairs, and RNA can also form such pairs⁹. Conceivably, such a folded structure could be recognized by the editing machinery.

How did cryptogenes arise? The similarity between the amino-acid sequence encoded by the final edited mRNA in a kinetoplast and the counterpart protein in other species argues for a common ancestor gene. Thus, the cryptogene seems more likely to be a derived form of the gene. In one possible scheme, the single ancestor gene, or a segment of it, was duplicated either as DNA or RNA, and one copy was transposed onto either maxicircles or minicircles to form short guide RNA genes (the inverted repeats at the ends of the guide RNA-encoding segments⁶ could be relics of such transposi-

tion). An RNA form of the other gene copy became the cryptogene through the same type of deletions and additions of U residues as in modern RNA editing, except that the equilibrium was heavily weighted toward deletions rather than additions of U residues.

RNA editing remains enigmatic, evoking the impression that it is a clue to something we may somehow have missed. When the mechanism is elucidated by the use of *in vitro* systems, it will perhaps provide insights into the evolutionary origin and role of this unconventional use of the genetic material. □

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Boris N. Veprintsev (1928–1990)

THE death, on April 11 of Boris N. Veprintsev robbed Russia of one of its true intellectuals. Professionally a zoologist and biophysicist, he was also much engaged in political activity. His life reflected much of the tragedy of modern Russia. His father Nikolay was a professional revolutionary who joined the Russian Social-Democratic Party (the Bolsheviks) in 1903. He suffered twice from repressions after the Great October Revolution and died in exile in 1941. From Nikolay, Veprintsev inherited both a hot temper and courage.

Arrested in 1951, Veprintsev suffered the devastating conditions of prison and exile together with such outstanding people as L. N. Gumilev, M. I. Kazanin and L. A. Voznesensky. He was freed in 1954, and returned to Moscow University, where he had been an undergraduate, to study the biophysics of membranes, specifically those of axons of ventral nerve cords from rainworms. Later, with the biologist D. A. Sakharov, he extended his studies to the giant neurons of brain ganglia of nudibranchs. And, at the laboratory of biophysics of the nerve cell at the Institute of Biophysics in Puschino, near Moscow — which he founded in 1963 — he initiated experiments on *in vitro* cultivation of nerve cells, studies on choline receptors, and on correlation between biochemical processes and electrical activity, function and plasticity of nerve cells.

But zoology and conservation had always ranked equal with biophysics for Veprintsev — his recordings of birdsong from Russia are well known to ornithologists. For many years, he promoted the idea of genome conservation, which originally seemed quite fantastic to many people. The idea was to maintain the frozen gametes and embryos of endangered species so that it would be possible to restore them in the future.

Presented by Veprintsev and N. N. Rott at the International Congress of the All-World Organization of Nature Conservation in Ashkhabad in 1975, the idea was supported by many scientists, Sir Peter Scott among them. Veprintsev was elected Chairman of the International Committee on the Conservation of Genetic Resources, in which position he stimulated collaboration between Soviet and foreign specialists. But it was only in 1989 that he was able to meet colleagues abroad. The new plans, hopes, energy and enthusiasm developed on these trips were broken by the severe disease that soon led to his untimely death.

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Astronomical glasnost

Bruce Morgan

BULGARIA is not often viewed by Western astronomers as central to mainstream research. But on page 637 of this issue¹, Tomov *et al.* report fascinating new observations of the odd star MWC560, an object first listed in the Mount Wilson Catalogue nearly 50 years ago², although it was never studied in detail. Their discovery stands as a reminder that even bright stars may still hide truly odd behaviour, merely awaiting a spectroscopist sufficiently interested to obtain a single modern observation. Despite the arsenal of complex electronic solid-state detectors in use at most telescopes, these new observations were made with the most classical of techniques, the photographic plate.

The spectra described by Tomov *et al.* indicate the presence of a red giant star, plus an additional source of excitation which causes intense emission lines to be superposed on the spectrum. The integrated light output of the star is also erratically variable on timescales extending from days to years. All of these characteristics are shared by a class of binary system known as the symbiotic stars, of which more than a hundred are known. These systems are thought to consist of a red giant plus a highly evolved white dwarf, which is too faint to be seen directly, but causes the emission lines by inducing mass transfer through an accretion disk.

The unique aspect of MWC560 now pointed out is that the hydrogen emission lines are accompanied by prominent, violet-shifted absorption lines. The Doppler shifts of the absorptions indicate that the gas velocities vary from night to night, and can grow as large as 6,000 km s⁻¹. The profiles of the absorption lines are complex, and also vary erratically. Similar spectral and photometric observations were reported several years ago at a meeting³, but failed to draw anyone's attention, perhaps as they were never published in a journal. The Bulgarian report will not suffer a similar fate. Already extensive satellite observations of the ultraviolet spectrum of the star have been submitted for publication⁴, again indicating erratic, very high velocity ejection of matter.

It is too early to form a coherent model of this odd behaviour, but all of the observers^{1,3,4} suggest that there is mass transferred from the giant onto a compact companion, followed by ejection of this matter from the system, thus explaining the high-velocity spectral absorption features. However, the peak velocities observed are far higher than those seen in symbiotic stars, or even in the far more spectacular nova phenomenon. Why MWC560 is uniquely different is the prize question — perhaps the nature of the

secondary star will bear on the answer.

The story of MWC560 carries some poignancy owing to the recent death of Nicholas Sanduleak, formerly of Case Western Reserve University in Ohio. Sanduleak spent a lifetime of work patiently surveying and cataloguing peculiar stars: the bizarre relativistic object SS433 and the progenitor of the 1987 supernova in the Large Magellanic Cloud, Sk -69° 202, both bear his name as the initial cataloguer. Just three weeks before his untimely death, Sanduleak, who was also responsible for one of the earliest spectral comments⁵ on MWC560, submitted a

GENOME MAPPING

Clone maps made simple

Peter Little

IF the human genome project is to reach its ultimate goal of establishing the DNA sequence of the entire 3×10^9 base pairs of human DNA, it will need to use cloned DNA 'maps' to break the genome up into manageable chunks. These maps are collections of recombinant DNAs that are aligned in an overlapping fashion, such that the whole genome is represented in the clone arrays and that any individual DNA base is contained in one or more clones (*a* in the figure). Hans Lehrach and colleagues now report¹ the first practical results of a method that Lehrach has been developing for the past seven years which may be powerful enough to generate large maps of whole genomes in a relatively non-labour-intensive fashion.

What is the method? Consider a random, short DNA sequence of *N* bases. One would expect this to occur once in 4^N bases (assuming all four bases occur with equal probability). As the sequence gets longer the frequency of occurrence must decrease and in principle it is possible to select a length and sequence of DNA, generally of around 10 bases that should be found every 40,000 base pairs or so, such that there is about a 50 per cent probability of any 40,000-base-pair fragment containing the sequence. Now consider two different DNA fragments cloned in cosmid vectors — these contain 40,000 base pairs of passenger DNA. The probability that a given sequence occurs at random in both of these cosmids is about 25 per cent but the probability of two unrelated DNAs having 10 or 20 sequences in common is tiny. We can build up a signature of presence or absence of sequences in each cosmid that could be presented as a binary string — 1 for present, 0 for absent.

circular to the International Astronomical Union⁶ discussing observations obtained a few weeks before, and motivated by an early announcement of the work by Tomov *et al.* It was Sanduleak's final contribution to the literature. Although he was not the very first to call attention to MWC560, this object will most certainly prove to be his kind of star. □

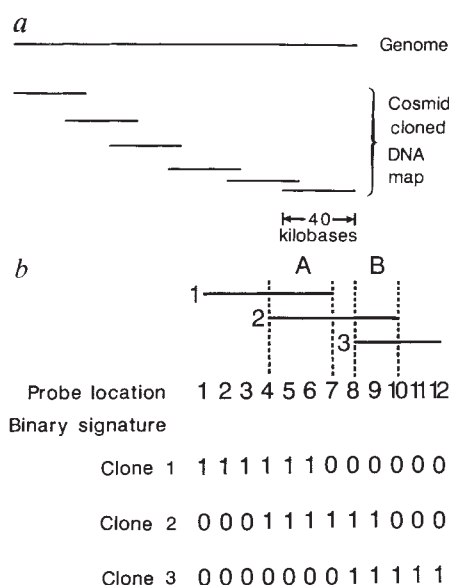
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Now consider the case if our two chosen cosmids overlap in the DNA they contain. Clearly, they will contain a similar pattern of sequences (*b* in the figure). Cosmids 1 and 2 overlap in region A and cosmids 2 and 3 overlap in B and therefore will have identical sequences in these regions. As long as there are sufficient test sequences, the overlap will be above statistical noise (that is, the probability of random similarity of occurrence patterns will be negligible) and we can generate the map of the three cosmids shown.

This scheme can be practically applied by using short DNA oligonucleotides as hybridization probes to cosmid clones: this is what Craig *et al.*¹ have done, using the 153-kilobase herpes simplex virus (HSV) genome as a model system. They used 22 12-base probe sequences from known positions in the completely sequenced HSV DNA and hybridized these to arrays of 384 cosmids. Although the probes were in defined positions in the genome, the analysis does not use this information. Instead, the 'binary' signature of each cosmid is analysed by computer programs that try to work out an optimum order for the probes by consideration of potential overlapping clone signatures, based on both presence and absence of sequences in clones. Thus the final solution to the analysis is both a map of cosmid clones and also a map of probe locations. The method seems to work well, and noise levels, which comprise cross-hybridization to related sequences, actual repeats of sequences, and spurious artefacts, seem to be well within the error limits.

What are the prospects for larger application of the technique? Lehrach has presented random-number simulations²



a, Cloned DNA map of a region of a genome — a complete map of the human genome would be represented by about 150,000 cosmids. **b**, Principle of the hybridization method. Three overlapping clones have the binary signatures shown. Because in this case the order and location of probes 1 to 12 is shown, the binary signatures obviously overlap. In practice, computers are required to determine the order of the probes, and location is given by overlap position.

showing that the method can be applied to the entire human genome, contained as robot-generated arrays of clones on 80–100 filters hybridized with 100–200 probes. The optimal frequency of probe hybridization is 30–50 per cent of cosmids hybridizing per probe, but it has proved difficult to generate this frequency — signal levels and specificity are problematic, and in practice it is not possible to predict easily the behaviour of an oligonucleotide of defined length from its sequence. This makes the construction of experiments more complex, but Craig *et al.* point out that hybridization frequencies of even 2 per cent can generate significant information.

There is clearly a long way to go and there is no doubt in my mind that the main technical problem is to identify usable sequences and to generate an adequate signal. The inability of cosmid and phage vectors to clone all eukaryotic DNAs is a further problem, but one that is shared by all cloning approaches. The new technique is testable and controllable, and the data so far suggest that its application to smaller genomes and whole human chromosomes is realistic. Lehrach (personal communication) has used 60 selected probes derived from simple repeat sequences to hybridize to *Schizosaccharomyces pombe* cosmid arrays, and has achieved a mean hybridization frequency of 6.1 per cent — certainly adequate for the analysis of this genome. The method can equally well be applied to complementary DNA libraries

to identify exemplars of every sequence in the messenger RNA population.

Four groups are using various technologies to try to map whole genomes. Kohara *et al.*³ and Olson *et al.*⁴, using slightly different methods, generated maps based on restriction-enzyme cleavage sites of randomly selected phage clones containing *Escherichia coli* or yeast DNA and constructed cloned DNA maps by alignment of restriction sites. Coulson *et al.*⁵ used the patterns of fragments generated by restriction-enzyme digestion of random cosmid clones, a process termed fingerprinting, to deduce statistical similarity between pairs of clones, and then constructed maps by alignment of pairs. This method is already being applied by us⁶ to regions of human chromosome 11 and has been modified by Carrano *et al.*⁷ to map chromosome 19. Evans *et al.*⁸ established the overlap of clones by using direct hybridization of clones with probes specific for the ends of other cloned DNAs. All four methods, however, generate limited information per individual analysis, whereas the main attraction of the Leh-

IONOSPHERE

Disturbing effects of radio

T. R. Robinson

THE inadvertent effect that human activity is having on the Earth's greenhouse blanket, in the lowest 10 km of the atmosphere, is much commented on. The effect we can have on the outer ionized regions of the atmosphere, over 70 km above us, is less discussed. Nevertheless, powerful radiowaves can interact in interesting ways with the ionosphere, and U.S. Inan has recently exploited this fact to conduct experiments simulating the effects of lightning discharges on the electrical properties of the upper atmosphere¹. A further development, of a much more speculative nature, is the proposal to use such effects to protect the ozone layer.

The effects of powerful radio emissions on the ionosphere are rather well understood, to the extent that they can be used to control the behaviour of some important characteristics of the atmosphere. Since the early 1970s, numerous controlled experiments have been carried out in the United States, the Soviet Union and Northern Scandinavia to investigate a wide variety of phenomena arising out of the interaction between radio waves and this ionized region of the upper atmosphere. Inan describes in his new paper an experiment in which the amplitude of a powerful radio transmitter operating at a frequency of 28.5 kHz was modulated with a 5-second cycle. During each successive 5-second period the

transmitter was kept on for 3 seconds then off for 2 seconds. The effects of this perturbing wave on the lower ionosphere (between 70 and 100 km altitude) were then inferred from the amplitude variations it induced in a 'test' wave propagated through the modified ionosphere on a completely separate, remotely located radio link operating at 24 kHz.

The interaction of two waves in this manner is termed cross-modulation. The very first ionospheric observations of this type of effect were reported in the pages of this journal in 1933, soon after the high-power transmitter at Luxemburg began commercial broadcasting². This so-called Luxemburg effect was explained almost immediately by Bailey and Martyn³, in terms of the heating of ionospheric electrons by the collisional absorption of radio waves. This same basic idea can also explain Inan's observations. The physical mechanisms involved are as follows. Electromagnetic waves which propagate through the ionosphere cause free ionospheric electrons to oscillate. As the electrons move under the influence of the wave they also collide with the numerous background neutral atoms. Energy is lost from the electromagnetic wave to the electrons, whose temperature consequently rises, and this in turn causes an increase in the temperature-dependent collision rate. So for a perturbing wave of sufficiently high

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power, this modification of the collision rate leads to a substantial further increase in its own absorption rate and also of the absorption of any other wave traversing the modified region. Thus, turning on the perturbing wave suppresses the test wave signal; turning the perturbing wave off restores the test wave signal to its unmodified value. The time constants involved in these changes are of the order a millisecond, so the test wave can respond almost instantaneously on the 5-second timescale used in the experiment reported by Inan.

Inan's experiment is significant in that the frequencies involved are the lowest at which cross-modulation effects have yet been observed. The original Luxemburg observations, for instance, were carried out with a 252 kHz perturbing wave and a 652 kHz (Beromunster) test wave. Wave generation and propagation characteristics at the lower frequencies are quite different from those for the usual radio modes. Inan suggests that one of the whistler modes — ionospheric disturbances that are audible in radio communications — is efficiently excited by his 28.5 kHz high-power transmitter and that this mode is strongly absorbed at about 85 km altitude. From his calculations Inan estimates that the heating effect resulting from the absorption of whistler wave would cause a 30 per cent change in the collision rate. This would account for the observed cross-modulation levels. Whistler waves in the tens-of-kilohertz frequency band, with wave amplitudes of up to 50 m V m^{-1} , are known to be excited by lightning discharges⁴ and Inan argues that these waves could produce similar direct changes in the ionosphere, albeit of a transient and local nature.

There is a further important effect caused by heating the lower ionosphere by high-power radio waves, the stimulation of increased levels of ionization concentration⁵. This arises because the recombination rate of electrons and ions decreases with increasing electron temperature. As is well known, the electron concentration in the lower ionosphere is determined by the balance between the production of electrons by solar photoionization and chemical recombination processes. A reduction in the recombination rate due to this heating engenders a build up of electrons. The change in the collision rate together with the electron concentration result in a change in the electrical resistance of the ionosphere at heights where currents of natural origin flow, and hence the strength of the currents are affected by the heating process. These currents are particularly strong at high geographic latitudes within the auroral zone, where the well known auroral displays, which also owe their origin to ionospheric currents, occur.

Many experiments in which radio

waves have been used to control auroral current flow have been successfully carried out with the high-power radio facility built by the Max-Planck-Institut für Aeronomie, Lindau, at Tromsø in Northern Norway⁶. But all of the Tromsø experiments have involved transmissions at frequencies of several megahertz. On the basis of Inan's results one can speculate that strong radio transmissions over a much wider range of radio frequencies than previously thought can control ionospheric currents. Moreover, it is also possible that lightning discharges may provide a mechanism by which the upper and lower regions of the atmosphere are coupled.

Returning finally to a topic more obviously environmental, A. Y. Wong *et al.* have suggested that powerful radio transmitters such as that at Tromsø may help to conserve ozone⁷. These authors point out that raising the electron temperature and density through radio heating in the upper atmosphere causes an increase in

the rate of conversion of neutral chlorine into negatively ionized chlorine. Unlike neutral chlorine, which participates in the well known chemical reactions which lead to ozone destruction, chlorine ions react only rarely with ozone. Wong *et al.* have proposed a trial experiment, for the near future, with a high power facility in Alaska. If their experiment were to prove successful, the question would then arise as to whether lightning discharges might also act to conserve ozone. □

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NUCLEIC ACID STRUCTURE

Tetraloops and RNA folding

Olke C. Uhlenbeck

THOSE interested in the function of RNA have long been frustrated by the lack of high-resolution structures. Much of what is known about RNA folding is based on the X-ray crystal structures of four rather similar transfer RNAs (tRNAs) and a handful of small oligonucleotides. The paper by Cheong *et al.* on page 680 of this issue¹ ushers in a new era of RNA structural biology: the authors use nuclear magnetic resonance methods, originally used for proteins, to determine the structure of a hairpin domain that occurs frequently in RNA.

It is well known that the predominant element of RNA secondary structure is the hairpin. Hairpins are formed when a local region of the polynucleotide chain folds back on itself to form a short, intramolecular base-paired helix, called the stem. The number of unpaired loop residues enclosed by the stem can vary substantially depending on the type of RNA. For example, most hairpins in tRNAs contain seven loop residues whereas the predominant number in the ribosomal RNAs (rRNAs) is four. This preference for certain loop sizes may reflect either thermodynamic² or structural³ constraints on reversing the direction of the RNA chain.

In their classic work^{4,5} using phylogenetic sequence comparison to define the secondary structure of the large and small rRNAs, Noller, Woese and co-workers noted a strong preference for certain sequences among the four-residue loops (tetraloops). Although 256 differ-

ent tetraloop sequences are possible, nearly 70 per cent of all the tetraloops in rRNAs are either UNCG or GNRA (where N is any nucleotide and R is a purine), and in several cases the entire tetraloop acts as a unit. Thus, a certain rRNA hairpin in one organism would have the sequence UUCG whereas that of the same hairpin in a closely related organism would be GCAA. Noller and colleagues suggested that both sequences form a particular, perhaps similar, loop structure. This conclusion is supported by chemical modification experiments⁶ on rRNA indicating that the variable second nucleotide in each tetraloop is more reactive than the remaining three conserved nucleotides.

The UNCG and GNRA hairpins occur frequently in many other RNAs. The 7S RNA in the eukaryotic signal-recognition particle contains two GAAA hairpins that interact with a protein⁷ and are essential for the function of the molecule⁸. The catalytic RNA of RNase P (ref. 9) and the self-splicing RNAs (refs 10, 11), also contain many hairpins of both classes. In the genome of bacteriophage T4, the sequence CUUCGG is restricted to intergenic regions where it appears as part of a hairpin. Tuerk *et al.*¹² noted that when reverse transcriptase encounters such a UUCG hairpin, it terminates a few nucleotides before copying the loop sequence. They suggested that UUCG hairpins must have a particularly stable structure that could not be disrupted by the enzyme. This prediction

was tested by showing that the thermal stability of isolated UUCG hairpins was substantially greater than several control hairpin sequences. GNRA hairpins are also exceptionally stable (J. Haney, unpublished data).

Cheong *et al.*¹ have now determined the high-resolution structure of a 12-nucleotide UUCG hairpin using NMR methods originally developed by Wuthrich¹³ for proteins. Instead of relying entirely on interproton distances supplied by nuclear Overhauser enhancement (NOE) build-up rates, estimates for three of the five backbone bond angles were obtained by measuring proton-proton scalar coupling constants. The authors used no less than 395 distance and angle constraints to calculate the final structure using distance geometry and constrained energy minimization methods¹. The resulting structure is in satisfactory agreement with the phylogenetic, thermodynamic and chemical modification data. Perhaps the greatest surprise is that a *syn* G-U base pair forms between the first and last residues of the loop, effectively making a loop of only two residues. To reverse the direction of the polynucleotide chain, the riboses of the two remaining single-stranded residues have an extended DNA-like C 2'-endo configuration. The phylogenetically variable and chemically reactive U residue protrudes into solution whereas the C residue is stacked inside the loop and makes an internal cross-loop hydrogen bond. Presumably this hydrogen bond and the extra base pair are responsible for the exceptional stability of the hairpin.

RNA biochemists have for a long time wanted to use computer folding algorithms to predict RNA secondary structures successfully. Nearly 20 years have passed since Tinoco and colleagues¹⁴ delineated a thermodynamic approach to the RNA folding problem. Essentially, they considered each RNA structure to contain a number of basic thermodynamic elements (helix stacking interactions, loops, bulged nucleotides and so on), each contributing a characteristic amount of free energy (positive or negative) to the overall stability of the molecule independent of the particular RNA in which they occurred. The authors then analysed the melting profiles of small model RNA oligonucleotides to deduce values of free energy for each element (the 'Tinoco rules'). Possible combinations of base-paired regions in a given RNA sequence were identified by computer and the total free energy calculated for each. The structure with the lowest free energy was then assumed to be the correct one.

Despite considerable improvements in the programs that find possible structures¹⁵ and a steadily increasing library of model compounds¹⁶, it is clear that the

method is not yet able to predict the structure of longer RNA molecules reliably. For example, several of the current computer algorithms did not successfully predict the secondary structure of *Bacillus subtilis* P RNA deduced by phylogenetic methods⁹. The basic problem is that many elements that either stabilize or destabilize RNA structure remain to be identified. These include long-range base-pairing interactions such as pseudoknots¹⁷, triple-stranded tertiary interactions of the type found in tRNA and, of course, unusual loop structures exemplified by the UNCG and GNRA tetraloops. Until these additional elements are identified and their thermodynamic contributions measured, the predicted structures will contain errors.

How much does the newly acquired knowledge of the thermodynamics of the UNCG and GNRA tetraloops aid in predicting secondary structure? An answer is provided by Jaeger *et al.*¹⁸ who assumed an extra 2 kcal mol⁻¹ for each of these structures and found a small but consistent improvement in their ability to predict phylogenetically proven RNA structures. Perhaps the experience with RNA tetraloops points the way to a synthesis of the more biological (phylogenetic) and chemical (thermodynamic) approaches to RNA structure. Chemists can solve the structures and measure the thermodynamic properties of units of RNA identified by biologists. The resulting more accurate folding algorithms will be invaluable for molecular biologists interested in the structures of mutant RNAs that cannot be solved by phylogenetic methods. □

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Currents of air

MODERN windmills, while a great improvement on their traditional ancestors, are still big and expensive monsters. They disfigure any landscape, and can cause television reception to flutter wildly. Daedalus now has a new approach.

The heated cathode of a radio valve is a copious emitter of electrons. What would happen, he wonders, if they were emitted not into a vacuum but into the air? They would immediately latch onto air molecules to form negative ions. Unlike electrons, ions can diffuse only slowly through the surrounding air. A wind blowing past the cathode would carry them away bodily, and could even 'pump' them against a considerable electric field. Quite a small valve-cathode can eject an amp of electrons, so a big mesh emitter erected on a windy site could launch a heavy current of ions. The wind would drive them to a parallel collecting mesh a few centimetres away. If the two electrodes were connected by a cable, the resulting return current would capture much of the energy of the wind blowing through the mesh.

This neat 'ionic windmill' has an obvious snag. The heated cathode of an evacuated valve consumes only a small fraction of the power conducted by the valve; but a large wind-blown heated emitter might well need almost all its captured power to keep itself hot. To make an energetic profit, the emitter may have to work in the cold. So Daedalus now intends to equip it with many small sharp discharge points, coated with materials of the lowest possible work function. He will charge the entire circuit to such a high negative voltage that the emitter grid sprays out ions copiously even at room temperature. Blown downwind to the rounded wires of the nearby collector grid, they will entrap wind energy with no needless thermal losses.

Even the best cold mesh emitter is unlikely to ionize more than about one in ten million of the air molecules blowing through it. A strong breeze might release a current of a few amps per square metre, and be able to blow it up a potential difference of 100 volts or so. Accordingly, a 100-metre-square ionic windmill could generate several megawatts of useful power.

Silent and unobtrusive, the ionic windmill will revolutionize the renewable energy business. Modern inverters and control electronics could easily convert its fluctuating output to a stable standard line voltage. It need not even rotate to face the wind; a static array in star or delta form could generate power in the most wildly veering breeze. It would be no more hazardous than any other high-voltage installation. And the inevitable leakage of negative ions into the air should subtly cheer up people living downwind.

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Mercury and crematorium chimneys

SIR—The possibility of injury to dentists and their patients from absorption of mercury associated with amalgam fillings has caused considerable concern. But several recent studies¹⁻³ have indicated that there is little risk associated with the current widespread use of silver/copper/tin amalgams⁴, apart from carelessness or with persons of exceptional sensitivity.

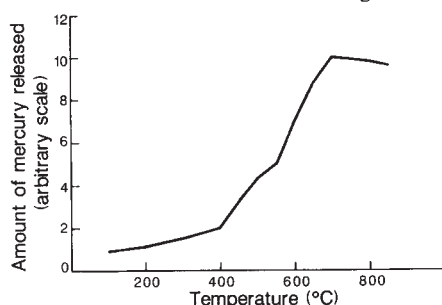
Unfortunately, none of the investigators seems to have considered the nature of the final disposal of the filled teeth and their owners. Burial should result in very slow and probably harmless release and dispersion of mercury, but the increasing use of cremation could lead to problems in view of the thermal instability of mercury alloys, the volatility of the free metal, its cumulative toxicity⁵, and the aggregate amounts now involved. Only the possibility of release of radioactive nuclides from implanted pacemakers and therapeutic devices has attracted comment⁶.

I have therefore investigated the thermal decomposition of dental amalgam with samples of well-aged alloy obtained from extracted teeth kindly provided by C. G. W. Wilks. Fragments were powdered, mixed with a little ignited alumina, and then placed, with a thermocouple, in a borosilicate glass test-tube. The tube was then closed with a rubber stopper pierced by a hypodermic needle and a narrow glass tube filled with glass wool coated with palladium chloride. The latter allowed pressures to equalize, but prevented any escape of mercury vapour into the laboratory atmosphere.

The contents of the main tube were then carefully heated with a flame, and equal small volumes of the contained air removed with a microsyringe at measured temperatures in a stepwise progression rising from ambient to 800 °C. The quantity of mercury vapour in each sample was analysed by T. Hackwell and myself by injection into a cold-vapour atomic absorption apparatus specific for this element⁷. A typical result is shown in the figure. It will be seen that decomposition of the dental amalgam was detectable at 200 °C, accelerated above 400 °C, and was essentially complete by 700 °C. Therefore, the contained mercury will be completely released by exposure of teeth containing standard dental fillings (BS 2938; 1985) to the temperatures involved in any form of cremation⁸. It is presumably carried as vapour in the hot gases blown from the chimney, with condensation and/or chemical combination occurring on cooling.

I have been unable to locate any figures for the average weight of mercury now carried by western adults in their fillings, but it is possible to derive an estimate from published statistical surveys⁹. These show that 30 per cent of adults in the United

Kingdom have lost all their normal complement of 32 teeth, with the rest having on average 7.5 filled — but otherwise satisfactory — teeth. In addition, there is a class for decayed teeth averaging 1.9 per head. Some of these will contain fillings that have failed in their primary purpose, but on the other hand 'fillings' will



Thermal decomposition of dental amalgam.

also include a proportion of the white mercury-free compositions used for front teeth. It seems reasonable to suggest an average of five amalgam fillings per head for the adult population of the United Kingdom. In 1988, more than 22 million such fillings were emplaced in England and Wales by National Health Service practitioners alone⁹. Even more were placed in previous years.

Nowadays, most dentists purchase commercially prepared sealed plastic ampoules containing encapsulated mercury and powdered alloy, and mechanically vibrate an ampoule of a suitable size to prepare a batch of amalgam. To obtain some idea of the amount of mercury

involved, I opened an unmixed ampoule of medium size and weighed its contents. It contained 0.6 g of metallic mercury and 0.6 g of silver-based alloy. Five amalgam fillings of average size would therefore contain 3 g mercury.

Local government records show that one city crematorium carried out an average of 3,723 cremations per annum over the past five years, rising to 3,831 in 1989. Simple multiplication suggests that around 11 kg of mercury is being released every year from this single (unfiltered) crematorium chimney. As the upper limit for long-term exposure of the general population to mercury vapour in the air has been proposed⁵ as only $1 \mu\text{g m}^{-3}$, it is imperative that a careful ground and air sampling programme be initiated to assess any possible hazard, and if necessary propose means (activated charcoal filters?) to ameliorate the situation. The safe disposal of extracted filled teeth (or, better, recovery of mercury and silver from them) should also be considered.

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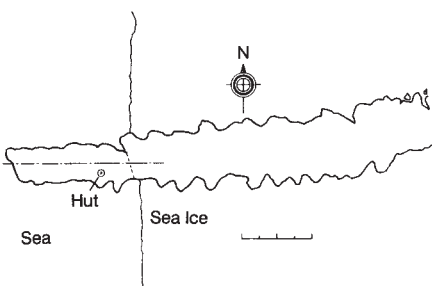
Calving of Erebus Glacier tongue

SIR—On 1 March 1990, a significant portion of the Erebus Glacier tongue calved. This tongue flows from the western side of Mt Erebus into McMurdo Sound, Antarctica, at a point about 20 km north of New Zealand's Scott Base. Before the calving event, the last 10 km of the tongue's 16-km length was floating and was, during most of the year, sur-

rounded by sea ice 1 to 3 m thick. The tongue's thickness varied from 50 m at the snout to 300 m at the point where it is grounded, while its width varied from 0.5 to 2 km with many large symmetric bays (see figure). The sea depth near the tongue's snout was 300 m.

The tongue was first observed to calve in March 1911 when members of Scott's British Antarctic (Terra Nova) Expedition of 1910–13 noticed that the last 4 km of the tongue had broken off during a gale. The resulting tabular iceberg drifted around in the McMurdo Sound for several months before becoming grounded on the western side of McMurdo Sound¹. A similar calving was deduced by Holdsworth to have occurred during the 1940s (ref. 2).

Recently, we were informed by the 'wintering over' staff at Scott Base that the Erebus Glacier tongue had on 1 March 1990 calved at the sharp bay near the hut containing our equipment used for monitoring the strain of the tongue over the past 5 years (see figure). A few days previously the sea ice surrounding the tongue



Schematic map of Erebus Glacier tongue showing the site of our hut and the edge of the sea ice on 1 March 1990. The dashed line across the tongue at the sharp bay is the line of fracture of the tongue. Scale bar, 2 km.

broke up to a point just east of the hut. At the time of the calving the wind was blowing at 10 knots down the length of the tongue and a heavy sea was running from the north. The resulting 100-million tonne iceberg of 3.5-km length was last seen floating past McMurdo Base with our hut on it. The sea near the glacier tongue refroze on 10 March, emphasizing the short time available for calving since the sea ice last broke back to such an extent about 10 years ago.

We believe the tongue calved because of two factors — first, and most important, the break-up of the surrounding sea ice made it vulnerable to the action of the sea and laterally far less rigid; and second, the tongue was weakened by the presence of a sharp bay, forming a stress concentration at the reduced section near the edge of the sea ice. The heavy sea coming through the entrance of McMurdo Sound in the north pushed the exposed length of the tongue in a southerly direction until it broke at the sharp bay just to the east of our hut (see figure). We would expect the next calving of the Erebus Glacier Tongue to occur around the years 2020–30.

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What's in a name?

SIR—Cedergren *et al.* in their Scientific Correspondence recently documented¹ discrepancies in terminology for apparently homologous genes in different species of bacteria. This is a general problem applicable to comparisons of genes among all species, as in most instances genes have been conserved both in structure and function throughout phylogeny.

The need for standardization of gene nomenclature in man was recognized at the Fourth International Workshop on Human Gene Mapping (Winnipeg, 1977) which established a nomenclature committee to formulate guidelines for an international system of human gene nomenclature. The original guidelines² have been updated at each subsequent workshop, with the last complete update being published as part of the proceedings of workshop 9 (ref. 3). The catalogue of mapped gene markers from the Human Gene Mapping 10.5 workshop³ will be updated in Oxford, United Kingdom, in September.

As part of its mandate, the nomenclature committee recommends on the symbols of all genes assigned to the human gene map. Since 1988 we have coordinated our activities with the International Committee for Standardized Genetic Nomenclature for Mice to ensure that homologous

symbols are used for homologous genes in the two species. Pre-publication services are provided routinely to several human genetics journals, to McKusick's *Mendelian Inheritance in Man*⁴ and its online version (OMIM) to disseminate information about the markers on the map to the scientific community.

The use of approved terminology for human genes also greatly facilitates the subsequent capture of data relevant to the human gene map by electronic databases such as the newly established Genome Data Base at the Johns Hopkins University. Investigators are urged to consult us before publication to develop gene symbols consistent with human gene nomenclature guidelines.

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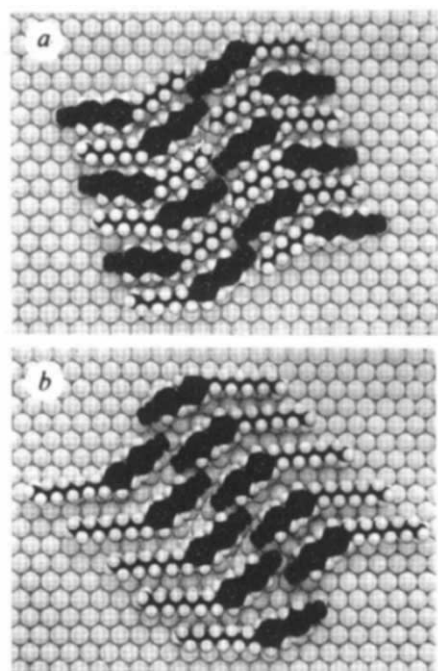
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Surface phases

SIR—Hara *et al.*¹ have shown, using scanning tunnelling microscopy (STM), that the smectic liquid crystal 4'-n-octyl-4-cyanobiphenyl (8CB) forms a completely different structure when adsorbed onto MoS₂ to that on to graphite. This result is striking because on graphite the family of liquid crystals 8CB, 10CB and 12CB all adopt a similar structure, the double-row or 'bilayer' structure in which cyanobiphenyl head groups are positioned next to other head groups and the resulting rows are slightly less than two molecular lengths in width^{2,3}. We have confirmed the result of Hara *et al.*¹ that 8CB on MoS₂ forms a single-row or 'monolayer' structure in which a head group always lies between two alkyl tail groups, resulting in rows approximately one molecular-length wide. But our STM results indicate a slightly different structure from theirs (*a* in the figure).

First, the structure is more symmetric — all heads and tails interdigitate. Second, we find the spacing between molecules to be 6.3 Å instead of 8 Å and



a, Model of 8CB on MoS₂ (the single-row structure) deduced from STM images of adsorbed molecules and the underlying substrate. The hydrogens in the alkyl tails register with the top-most sulphur layer. The molecules are separated by $2a_0=6.32$ Å and form rows of width $4\sqrt{3}a_0=21.9$ Å, where $a_0=3.16$ Å is the lattice spacing of MoS₂. *b*, Model of 10CB on MoS₂ (the double-row structure). The alkyl groups are all orientated to the MoS₂ lattice and are spaced $\sqrt{3}a_0=5.47$ Å apart. The biphenyls are spaced $2a_0=6.32$ Å apart.

the layer spacing to be 22 Å instead of 21 Å. We have measured the orientation of the underlying MoS₂ lattice to determine the registry of the molecules with the substrate, as was done previously for graphite². The molecules are aligned along one of the MoS₂ lattice vectors, confirming the proposal of Hara *et al.*¹ that heteroepitaxial growth occurs. We have also studied 10CB on MoS₂ (*b* in the figure) and find it to form the same double-row structure as it does on graphite^{2,3} but scaled to the larger lattice constant of MoS₂ (3.16 Å rather than 2.46 Å).

It is surprising that a change in the length of the alkyl group by only two carbons, from 8CB to 10CB, produces such a pronounced change in positional order on MoS₂. Why does 8CB form the double-row structure on graphite and the single-row structure on MoS₂? When lying flat, the biphenyl group is 5.9 Å wide, considerably larger than the 4.1 Å-wide alkyl group. The single-row structure therefore demands a separation between second-nearest-neighbour molecules of 10.0 Å. On graphite the closest packing that maintains registry with the substrate gives a separation of only $2\sqrt{3}a_0=8.52$ Å. The single-row structure on graphite would therefore require a separation of $3\sqrt{3}a_0=12.78$ Å, resulting in an inefficient packing structure with a high energy, because of the negative heat of adsorption

of alkanes on graphite¹. So on graphite the double-row structure is energetically favourable, allowing the alkyls to be separated by $\sqrt{3}a_0=4.26$ Å and the biphenyls by $2a_0=4.92$ Å. The latter can be accommodated if the biphenyls are somewhat tilted, but some strain remains, as evidenced by the dislocations that occur every fourth molecule in 8CB and every fifth molecule in 10CB^{2,3}. In contrast, on MoS₂ the closest packing of second-nearest-neighbour molecules is $2\sqrt{3}a_0=10.94$ Å. This larger separation can easily be accommodated by the single-row structure, in which a biphenyl group always lies between two alkyl groups. There is no crowding (*a* in figure) and no jumps in the rows as in the double-row structure. The single-row is probably the more energetically favourable as the cyano groups show strong dipole behaviour so that packing them close together (as in the double-row) leads to an increase in electrostatic energy.

Why then does not 10CB also form the single-row structure on MoS₂? We believe that the answer lies in the symmetry of

8CB: both the head and tail are 11.4 Å long, so heads and tails can alternate without leaving vacancies on the substrate. But 10CB has a 13.7-Å-long alkyl group, so a single-row structure would lead to energetically unfavourable vacancies. This argument predicts that other non-symmetric alkylcyanobiphenyls also will not form the single-row structure. We find that 12CB on MoS₂ does indeed form the double-row structure. We conclude that when molecules containing aliphatic groups wet the surface of layered chalcogenides, the resulting molecular lattice is determined principally by substrate registry, maximizing the number of substrate sites filled by the molecules.

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Twists and turns

SIR—Despite progress in elucidating the structure of ATPases that actively transport cations across cell membranes, the mechanism of this transport remains unknown. In the case of the calcium ATPase from the sarcoplasmic reticulum, the calcium-binding ability of the molecule has been suggested to occur mainly in the carboxy-terminal end^{1,2}. Clarke *et al.*¹ suggested that the amino-acid residues critical for calcium transport are inside the membrane, based on a prediction that there are ten intramembranous helices in the polypeptide chain of this ATPase. These residues could be clustered to form the calcium-binding site³.

But if there are fewer than ten intramembranous helices, the residues involved in translocation would have a different position with respect to the lipid membrane. For (Na⁺ + K⁺)ATPase, for example, a model containing only seven helices has been proposed⁴. Application of this model to the Ca²⁺ATPase indicates that residues critical for calcium translocation are all, with one exception, not intramembranous, but located either on the outer or inner aspect of the membrane. Because the hydrophathy profile and the release pattern of water-soluble peptide fragments are nearly identical for the carboxy-terminal half of both transport ATPases, it seems unlikely that their structures should differ fundamentally. In the case of the M5–M6 pair of helices, the existence of which is the most controversial, the dilemma is that although there is a long stretch of predominantly hydrophobic residues, this might nevertheless be too short to be accommodated

within two intramembranous helices, in accordance with the experimental evidence that part of this region is exposed¹. Furthermore, experiments with the gastric (H⁺ + K⁺)ATPase have been considered consistent with the location of the loop between M5 and M7 on the extracellular side⁵.

These contradictory experimental facts and the proposed folding patterns emphasize the difficulties in deducing membrane protein structure from amino-acid sequence alone. They also highlight the uncertainties about the basic features of ion translocation. If the amino acids essential for Ca²⁺-dependent phosphorylation are indeed placed on both sides of the membrane, they could form gates that are open only intermittently during calcium-ion translocation. But more experiments are needed to distinguish between the various possibilities.

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Iron solution

SIR—Martin *et al.* (*Nature* **345**, 156–158; 1990) in their study of the limitation of phytoplankton growth by iron deficiency conclude by suggesting that exogenous iron may stimulate growth to an extent which may be able to significantly affect the CO₂-induced greenhouse effect. Davies (*Nature* **345**, 114–115; 1990) indicates that perhaps 10⁶ tonnes of iron would be required to carry out this process.

I would like to suggest that an economic, readily available source of this mineral can be found in the manganese/iron concretions or nodules that are widely distributed in considerable quantity on the sea bed in the Pacific and other oceans. Nodules contain approximately 13% iron, 18% manganese and other trace elements such as nickel, copper and cobalt in lesser quantities. If they were to be collected and the metal content solubilized and released on site, this could provide an ideal mixture of trace elements for the enhancement of plankton growth.

It is interesting to speculate whether the oceanic depletion of these minerals by their adsorption onto nodules, to some extent regulates CO₂ incorporation into ocean biomass, in which case recycling would provide the ideal method for upregulating the whole process.

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Toxic sludge

SIR—In 1971 I began a study on the long-term consequences of a heavy application of sewage sludge on extractable levels of a range of trace elements in the soil and on uptake of these elements by herbage. I applied 150 tonnes per hectare of a commercially marketed dried sewage sludge to an unreplicated plot (25 square metres) and sowed perennial ryegrass on this area and on an adjacent control plot. I monitored the trace-element composition of soil and herbage in the control and treated plots at 6-monthly intervals for 15 years. (I have published fuller details of the sampling program, analytical methods and earlier results for this trial elsewhere^{1,2}.)

The composition of the sludge and of

TABLE 1 TRACE ELEMENTS IN SEWAGE SLUDGE AND IN TREATED AND UNTREATED SOILS

Extractant	EDTA		Total				
	Cu	Pb	Ni	Zn	Cd	Hg	
Sludge	181	710	430	3120	88	23	
	EDTA-extractable						Total
Soil, Sept. 86	Cu	Pb	Ni	Zn	Cd		
	C 3.8	14	1.6	6.8	0.27	0.88	
	T 37	51	3.5	48	0.63	1.3	

C, Untreated soil; T, treated. Units in µg per g dry matter.

TABLE 2 TRACE ELEMENTS IN HERBAGE FROM SLUDGE-TREATED AND UNTREATED PLOTS

	Cu	Pb	Ni	Zn	Cd
Sept. 86 C	7.3	0.76	0.77	20	0.14
T	8.2	1.1	1.6	55	0.23

soil from the control and treated plots in 1986 is given in Table 1. These results indicate substantial long-term contamination of the soil with respect to every element determined; contamination of top soil can persist for 15 years after a single application of sewage sludge. The effects of this contamination in this case are likely to be exacerbated by the final difference in pH between the soils from the control (pH 5.8) and sludge-treated (pH 5.1) plots.

The trace-element composition of the herbage grown on the control and treated areas sampled in 1986 is given in Table 2. These results indicate persistent significant enhancement of the levels of copper, lead, nickel, zinc and cadmium in herbage from the sludge-treated plot. Although none of these levels is hazardous, it is clear that there has been no significant reduction in availability of these metals over the 15-year period. This finding is in general agreement with other

reports³⁻⁶. Contamination of soils with a wide range of potentially toxic metals following application of sewage sludge is therefore virtually irreversible.

In view of the undesirability of entry of potentially toxic metals into food chains from contaminated soils, the ocean may well be the best long-term site for disposal of metal-contaminated sewage sludge. The total volume of the Earth's hydrosphere has been estimated by Park⁷ as 1.4×10^{18} cubic metres. The dilution potential of this enormous mass of water is ultimately an insurance against even serious abuses.

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Genetic linkage in mental illness

SIR—The recent articles¹⁻³ on genetic findings in mental disorders raise important issues regarding the interpretation of linkage results in complex diseases. Central to this debate is the question of what constitutes evidence for linkage in common disorders with non-mendelian transmission and phenotypic uncertainties. I would like to provide a broader perspective.

First, the lod score statistic (the logarithm of odds in favour of the linkage hypothesis) was devised primarily for rare mendelian diseases⁴. In the absence of prior clear evidence for major gene effects in mental disorders (segregation analysis, biochemical clues), and given the other complications discussed below, there is no theoretical foundation for designating a particular lod criterion as 'proof' of linkage (that is, a lod threshold high enough to make false-positives unlikely). But it stands to reason that the higher the lod the more likely it is that a given finding is correct. That is not to say that mendelian transmission may not exist in some subsets of mental illness — indeed, this is the basic tenet of linkage studies in psychiatry — but the evidence for linkage must be scrutinized closely.

Second, because of the complexity of these conditions, linkage analysis often involves several definitions of disease and, possibly, permutations of genetic parameters and model assumptions which can be varied to maximize, and thereby

inflate, the lod score (type I error). Thus, the confounding effect of testing multiple marker loci³ is but one among other encountered in multiple test models.

Third, conditions common in some mental disorders such as aetiological heterogeneity, diagnostic uncertainties and phenocopies, incomplete penetrance, misspecification of genetic parameters, assortative mating and other forms of bilineal transmission, and cohort effect, may lead to false negative linkages (type II error). False claims of non-linkage, including non-replication of positive linkage findings, are just as destructive to the field as type I errors.

What, then, is the solution? I would like to suggest some guidelines. (1) The conventional lod score approach may have to be augmented by other statistical methods such as empirical significance levels derived from the actual disease pedigrees. Further advance in linkage methodology and molecular biology techniques will be required to devise guidelines for optimal sample design in non-mendelian disorders, to unravel interaction among loci and to detect with confidence 'minor' locus effects. (2) Linkage results based on a narrowly defined phenotype with greater diagnostic reliability and presumed validity than broader disease categories may be accorded greater weight. (3) Analysis of strictly defined cases in which unaffecteds are assumed to have unknown phenotypes, thus obviating the question of

incomplete penetrance. (4) Introduction of quantitative clinical and/or biological covariates which grade stability and severity of the psychiatric diagnosis. Quantification of potential diagnostic errors could enhance the use and precision of linkage analysis. (These measures could also be instrumental in sorting out homogeneous illness subsets which could be more amenable to the linkage approach.) (5) When several diagnostic schemes and model specifications are used, they should be spelled out clearly, selected before the analysis and not be allowed to 'proliferate'. Some adjustments to multiple test effects have been proposed^{5,6}. (6) Priority should be given to pedigrees with no evidence of bilineal transmission. Also, to maximize the amount of potentially useful genetic information, simulation studies of pedigree structure and disease phenotypes could be used to determine *a priori* the pedigrees most suitable for the linkage analysis. (7) The reliability of the psychiatric diagnosis should be checked periodically to avert diagnostic 'drift'. (8) Marker typing and diagnostic assessment should be blinded with respect to each other.

These measures could lead to more consistent linkage results. Although independent replication is the ultimate gold standard, it may not be easy to come by in the near term, primarily because of aetiological heterogeneity. In the meantime, reported linkages can be subjected to further scrutiny by diagnostic follow-up (aimed at assessing new illness onset and diagnostic stability), extension of pedigrees, reanalysis of the data using stringent diagnostic criteria, and testing new marker loci in the candidate chromosomal region to confirm and refine the putative gene location. A case in point is the re-evaluation of the Amish data which has all but dashed the previously claimed linkage between bipolar affective disorders and chromosome 11p loci⁷.

It is perhaps unavoidable that the path toward the elucidation of genetic aetiology in mental disorders will be marked by fits and starts. My proposed guidelines may render the task at hand less convoluted. Some of these issues have been discussed in detail elsewhere⁸⁻¹⁰.

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Skeletons on the coffee table

Ralph A. Lewin

The Diatoms. Biology and Morphology of the Genera. By F. E. Round, R. M. Crawford and D. G. Mann. Cambridge University Press: 1990. Pp. 747. £125, \$250.

DIATOMS are a Johnny-come-lately class of microscopic algae, having evolved at about the same time as flowering plants, birds and mammals at the end of the Jurassic. We can be reasonably sure of this because diatoms, like birds and mammals, have characteristic mineralized skeletons, generally constituting a considerable proportion of their weight. These can make readily identifiable fossils. By contrast to our own internal skeletons, which are phosphatic, those of diatoms are siliceous, being initially composed of amorphous opalite and then, if they don't dissolve, changing slowly to microcrystalline quartzite as they fossilize. But the ephemeral substance of diatoms is, like ours, based on carbon; not even these organisms can make organosilicon compounds like silicones. Their shells have aesthetically pleasing, radially or bilaterally symmetrical patterns of pores and grooves, pits and prominences, many being reminiscent of lace doyleys. It is on these microscopically revealed details that the classification of diatoms has been based. Tens of thousands of species have been identified, described and figured, almost exclusively on the basis of skeletal patterns, some revealed by good optical microscopes and most now, *a fortiori*, by electron microscopy.

Traditionally, the first thing a diatom taxonomist does when he wants to identify a specimen is to 'clean' it by burning away or otherwise destroying all of its organic substance, leaving only its skeletonized remains for critical microscopic examination. One could conceivably have developed a diatom taxonomy based on whole, living cells — perhaps by using cytological, physiological and biochemical features, as has been done for other kinds of microbes — but I suspect that only a small proportion of the species now recognized would have been distinguishable in this way. Today, such a classification of the Bacillariophyta

would be unthinkable.

Why am I telling you all this? Because we now have a lovely new book on diatoms, impressive in its heft, magnificent in its production, encyclopaedic in its coverage and elegant in its illustrations. About 90 per cent of it deals with diatoms' skeletons, illustrated by 5,000 (!) electron

IMAGE
UNAVAILABLE
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REASONS

Beautiful examples of the intricately patterned, glass-like frustule (cell wall) of diatom species.

micrographs (mostly scanning) that show with exemplary clarity representatives of virtually every known Recent or fossil genus of diatoms. Each genus is assigned a pair of opposed pages, largely filled with photographs — about ten for each. I note many original features. For instance, most species in the old and unwieldy genus *Navicula* have been here reassigned to a score or more of new genera, partly on bases of soft-part differences, some suggested by Mereshkovsky in the early 1900s.

As explained in the introduction — for reasons that I consider unacceptable — no indications of scale are given, though the magnifications range over at least four orders of magnitude. (Of course, overall dimensions of diatom shells vary, but — as stated and illustrated in the text — stria and punctum spacings tend to be much less variable. And, of course, calibrations of electron microscopes can constitute sources of errors. But I think that any scales would have been better than none at all.) This doesn't detract from the aesthetic appeal of the micrographs, but it certainly reduces their scientific value.

The text carries a profusion of recondite details about the organisms' fine structures, both to delight diatom devotees and to enchant the artistic laity. There are many diagrams, most beautifully executed. (It's a pity that the editors couldn't have put the legends for Figs 60 and 61 on facing pages instead of overleaf.) A few small misstatements about the ecology of marine planktic diatoms presumably reflect the predominantly limnological and palaeontological experiences of the authors. Though the book is full of italicized generic and specific names, I found none misspelled — surely an editorial feat. This is clearly the culmination of decades of the authors' care and devotion to diatomology.

What of the first, textual 130 pages, dealing largely with diatoms as living organisms, respiring and photosynthesizing, floating as plankton, gliding on mud or sticking to solid substrata like the water weeds and rocks on river banks and sea shores? What do we learn about their specialized polysaccharides, pigments and oils, or their arcane systems of sexual or asexual reproduction? Rather less than from a 500-page volume on diatoms, edited by Werner and published in 1977. But the style of Round *et al.* is more consistent, its illustrations (of plastids and various fine structural details of cell organelles, and so on) are more abundant, and of course much of its information is more up to date. As a reference work on diatoms as living organisms it may be somewhat less comprehensive, but as a text it seems more readable.

There is a 29-page appendix with formal descriptions of new orders, families, genera and species. (If you think that Latin is a completely dead language, you should scan this section and think again.) There is another appendix of 24 pages listing all diatom genera with their authorities, as compiled by Ross and Sims. And there is also a separate, comprehensive index of genera and species. The authors have not included a glossary, but I think this doesn't matter much as almost all of the specialized terms (*rimoportulae*, *fultoportulae*, *copulae*, *nodulae*, *pseudocelli*, *helictoglossae*, and so on) are explained in the text and referenced in the subject index. Finally, there is a full bibliography of about 800

references, about half of which were published after the completion of Werner's book.

If you can afford it, buy a copy. (If you are wealthy, buy two — a second for your coffee table, to entrance friends and relatives.) If you can't, see that your librarian does so. (And if not, maybe you should find a more compliant librarian.) □

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Causes and cures

Stephen Cotgrove

Post Environmentalism. By John Young. *Belhaven: 1990. Pp. 225. £25. To be published in the United States in October by Harvard University Press with the title Sustaining the Earth, price \$19.95.*

ACID rain, the greenhouse effect, pollution, the exhaustion of fossil fuels, the population explosion — all these threats to the future of human existence can be laid at the door of science and technology. Such an accusation is, of course, simplistic. The uses to which scientific knowledge is put, the increasing control of the direction of research by governments and, behind these, the demands of the industrial-military complex for useful and relevant research, are more appropriate concerns. To rely on technological fixes from the environmental sciences will fail to get to the roots of the problem, which are embedded in the nature of industrial societies, and are fundamentally economic, political and moral.

John Young explores the extensive literature generated by the growing awareness of the environmental crisis in a search for its causes and cures. To sharpen the perspective, there is an account of some societies which have maintained a sustainable relationship with nature. "The genius of those peoples who have retained some of their land but also their ethos has lain in their ability to select from the experience of Western invasion and colonization, those aspects of our value system which reinforce their own, such as stewardship, reciprocity, family loyalty, community, service and charity, but to reject such goals as the accumulation of individual wealth as an end in itself, and ideas such as the separation of humanity from the natural world, which industrial society had developed" (page 49).

These, of course, are not the core values of industrial societies: they are certainly not the values of the marketplace. To identify these as the values crucial for a sustainable society is to underline the magnitude of the problem. And it is here that the writings of environmentalists are

so often unconvincing. Attitudes and values are remarkably resistant to change. And some are deeply embedded in Western culture. Young, for example, gives a more detailed account than most of the Christian tradition in which Genesis commands man to "be fruitful and multiply, and replenish the earth and subdue it, and have dominion over..... every living thing". Although the tradition also includes the notion of stewardship, the general thrust has been to legitimize mankind's exploitation of nature. It is to recent secular philosophers and environmentalists that Young turns for a critique of material values and the assertion that man is a part of nature.

The writings of Schumacher are of particular interest. He traced the roots of consumerism and a disproportionate emphasis on material values to the alienating technologies of production, which destroy the human satisfaction of creative work, generating conditions that make workers on assembly lines and in giant administrative and service bureaucracies easy prey to the fantasies of consumerist advertising. Hence industrial societies are firmly anchored to the treadmill of economic growth to buy compensation for unfulfilling work.

Young realistically recognizes that the "Green" constituency is a complex and heterogeneous conglomerate of radicals and reformers. Despite this, he finds hope in the emergence of common ground which challenges "the right of either profit-based capitalism or growth-oriented socialism to do what it likes with an important part of nature" (page 145). He finds grounds for optimism, too, in those exemplary initiatives by local governments in job creation, urban farms, public transport, recycling, and in developing skills which enable people to employ themselves. Whether such policies can achieve the profound transformations required to bring world population and exponential economic growth under control is a matter for scepticism.

As a contribution to the search for a more environmentally friendly culture, this book is interesting and useful. But for more down-to-earth guidance on causes and cures, one needs to look elsewhere, for example to Alan Schnaiberg's *The Environment from Surplus to Scarcity* (Oxford University Press, 1980), with its more rigorous analysis of the role of economic and political structures in the degradation of the environment. □

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■ Bill McKibben's *The End of Nature*, one of the most commercially successful of this year's environmental books, is published in Penguin paperback on 30 August. Price £4.99. (For review see *Nature* **344**, 885; 1990.)

Twistor magic

Edward Witten

Twistor Geometry And Field Theory. By R. S. Ward and Raymond O. Wells, Jr. *Cambridge University Press: 1990. Pp. 520. £50, \$79.50.*

Twistor theory, conceived about 20 years ago by Roger Penrose of Oxford University, is undoubtedly one of the most remarkable developments in mathematical physics in modern times. Penrose's achievement was to find a skilful way to reinterpret the coordinates of space-time as complex variables, while preserving the fundamental relativistic symmetries. The new complex variables that he introduced in this process he called twistors.

The original motivation for twistor theory was to find a new framework for the quantization of gravitation — for overcoming the longstanding incompatibility between general relativity, which is Einstein's theory of gravitation, and quantum field theory, which is the framework in which physicists have succeeded in understanding all the other forces. Many physicists have suspected that the concepts of continuum space-time, and of space-time points, would have to be abandoned to make sense of quantum gravity. Penrose suggested twistor space as the new concept that would replace space-time. To this day, one of the big mysteries is whether, and how, twistor theory will eventually fit into a theory of quantum gravity, perhaps in conjunction with some of the still raw ideas that have emerged from string theory.

Whatever the original motivation, the concrete successes of twistor theory, so far, have been in reinterpreting and often solving certain nonlinear classical field equations in four-dimensional space-time. In the mid 1970s, Penrose succeeded in interpreting the "self-dual Einstein equations" as integrability conditions for the existence of a "deformed twistor space". The self-dual Einstein equations, some of whose special properties had been first exhibited by J. Plebanski, are roughly speaking the equations for a gravitational field of one definite handedness or polarization. The twistor transform of the self-dual Einstein equations demonstrated that those equations were integrable equations, with miraculous properties similar to those of the celebrated soliton equations of two-dimensional mathematical physics. The self-dual Yang-Mills equations (equations that arise in modern gauge theories of the sub-nuclear particles) can also be solved by twistor methods, as was discovered soon afterwards by Richard Ward, one of the authors of this book and at the time a graduate student at Oxford University.

In the twistor interpretation of these equations, the third complex variable that Penrose had introduced to preserve the relativistic symmetries plays the role of the "spectral parameter" — the auxiliary variable used in solving those two-dimensional nonlinear equations that are miraculously soluble, like the KdV equations, the sine-Gordon equation, and so on. In fact, in the light of some of the more recent developments, like the work of L. Mason and G. Sparling deriving the nonlinear Schrödinger and KdV equations by "dimensional reduction" of the self-dual Yang-Mills equations, one is tempted to suspect that wherever there is a spectral parameter, there is twistor space lurking behind the scenes.

To understand twistor theory, one must have at least some knowledge of a remarkably diverse range of mathematical tools that have not been part of the standard repertoire of theoretical physicists. The authors of *Twistor Geometry and Field Theory*, Ward (now at Durham University) and Raymond O. Wells, Jr, of Rice University, have made a laudable and on the whole rather successful attempt to make their book self-contained. Following a first chapter which is devoted to the classical geometry of twistor space, they attempt in two long chapters — nearly 200 pages in all — to explain the techniques, mostly involving complex manifolds, fibre bundles and sheaf cohomology required for understanding the twistor transform of the self-dual Einstein and Yang-Mills equations. The next three, shorter chapters are intended as an introduction to classical

field theory for mathematicians. The authors finally arrive in part III of this book at the heart of the matter, which is the twistor transform of various linear and nonlinear field equations in four-dimensional space-time. They explain the basic ideas in part III very thoroughly and well. Their patience and care with the introductory material has unfortunately meant that some of the more advanced subjects in part III — like the detailed constructions of Yang-Mills instantons by M. F. Atiyah, V. G. Drinfeld, N. Hitchin and Yu. I. Manin, or the constructions of monopoles by Hitchin and W. Nahm — are only sketched. A second volume developing some of these matters in detail would still be valuable.

Twistor Geometry and Field Theory is in general skilfully written. It will serve as a relatively accessible introduction to twistor theory for many readers who have not studied the subject before. Others will find it useful as a refresher and as a source of many valuable insights. Moreover, the book appears at a fortuitous time. Because of developments in string theory, the mathematical techniques required in twistor theory are not nearly as alien to theoretical physicists as they were 10 or 20 years ago. Meanwhile, the theoretical physics community may be ready to think in a new way about the big riddle of how twistors can be used in quantum theory. □

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Rich sequel

Thomas D. Pollard

Molecular Cell Biology. 2nd edn. By James Darnell, Harvey Lodish and David Baltimore. *Scientific American Books*: 1990. Pp. 1105. Hbk £49.95, \$52.95; pbk £25.95, \$42.95.

THE authors of *Molecular Cell Biology* have thoroughly revised and beautifully illustrated this second edition, continuing their success in presenting a comprehensive, integrated account of fundamental life processes at the molecular and cellular level. The book is comprehensive in that it covers viruses, bacteria and simple eukaryotes as well as animals and plants. It is integrated in two ways. First, general principles are highlighted by presenting in parallel a diversity of specialized solutions to common needs such as RNA synthesis. Second, each section is integrated by weaving together biochemistry, genetics, molecular biology and morphology with physiology, developmental biology and even some pathology.

This vertical and horizontal integration reflects how biological research is done today, but precludes thorough coverage of most things even in 1,076 pages. The book contains enough biochemistry, molecular biology and genetics for the reader to understand the fundamentals of these subjects, but it cannot stand on its own as a biochemistry or genetics textbook. Developmental biology is featured in many sections, especially in that on the regulation of gene expression, but one cannot turn to this book to find out what it takes to make a fly. Consequently, the book may not fit comfortably into many traditional courses. But *Molecular Cell Biology* has a different mission. It represents the contemporary way of thinking about biology, a point of view where yeast mating types, inositol phospholipids, *Drosophila* homeotic genes, ion channels, zinc fingers and microtubule motors contribute on an equal footing to our appreciation of life processes.

Visually, *Molecular Cell Biology* is striking as it is the first book of its kind to use full-colour illustrations throughout, which is helpful for molecular models, light micrographs and many diagrams. But it can be overdone — microtubules appear pink, red, orange, purple and blue on successive pages! Regrettably, some illustrations are misleading because molecular components are not drawn to scale.

I enjoyed reading *Molecular Cell Biology*, but beginners will have to work hard because the text and figures are so rich in information. Teachers will also have to provide considerable guidance, because even in this big book it has not been possible to provide completely lucid



Not a modern problem — the high street open sewer of a mediaeval city as portrayed in *River Pollution: An Ecological Perspective* by S. M. Haslam. The volume analyses the biological impact of pollution and discusses methods of measuring and monitoring pollution damage. Published by Belhaven, price is £47. □

descriptions of many things (see, for example, the description of fluorescence microscopy).

One gripe is that the authors have, for the most part, left out the one thing that nonexperts can never know — what is not known. An expert will find examples on every page where the facts are not yet in or are contradictory. Yet phrases like “still not known” and “unclear how” are few and far between. A textbook writer cannot leave a trail of uncertainty for a novice reader, but the next generation of biologists deserves to know more about the soft spots in our knowledge, if only to provide food for thought about future contributions. One simple way to do this would be to distinguish more clearly which illustrations are models and which are established facts.

The inevitable question is how does *Molecular Cell Biology II* compare with *Molecular Biology of the Cell II* by Alberts *et al.* (Garland, 1989)? Both are authori-

tative, up to date and beautifully produced. The latter is more consistent in approach and style, and its authors make more effort to point out gaps in our knowledge. It is 13 per cent longer and packs more detailed information into its diagrams. But the former makes a better job of integration across the biological kingdoms. One could build an excellent course around either book. As in art, the decision is a matter of taste. □

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■ For review of the first edition, see *Nature* **323**, 401; 1986. *Molecular Biology of the Cell* was reviewed in *Nature* **302**, 637; 1983 and its second edition in *Nature* **340**, 608; 1989.

■ Published on 10 August is *A Student's Companion to Molecular Cell Biology*, by D. Rintoul *et al.* Again, publisher is Scientific American Books; price is £14.95, \$17.95 pbk.

A useful addition

Harry Cotton

Computing Before Computers. Edited by William Aspray. Iowa State University Press: 1990. Pp. 266. \$27.95.

IF YOUR grasp of computer history starts with microcomputers, this book should demonstrate that there was computing life before electronic digital machines. Others whose knowledge is broader will find here new topics along with the familiar. You may know about Roman numerals, but have you seen additions and multiplications fully worked through in them?

For many people, early computing means the abacus, logarithms, slide-rules and Babbage. They are all here. But there are surprises — the chapter on Babbage scotches the widely held belief that Ada Lovelace was the first programmer. It is implied that the work she did for Babbage was not original programming. If women programmers, trying to combat the male dominance in their profession, need a role-model, it may have to be the much later Grace Hopper of the US Navy (coiner of the term “bug”).

The period from Babbage until about 1950, which is often a blank in computing history even for computer people, is well documented in *Computing Before Computing*. One example is the discussion of why valves did not replace relays at an earlier stage in the design of computing equipment. It is paradoxical that a device as inherently unreliable as a valve should ultimately supersede the more robust relay. By running valves at well below their limits and never switching off their

heaters, reliability was improved. Valves usually burn out totally so engineers know when there is something wrong and where the correction has to be made. Finally, the greatly increased speed of operation of valve equipment over relays won the day. The rule about not switching off valve machines was not well-learned, however, as the first programmers found when they faced a digital computer on a Monday morning.

With five authors (including the editor) sharing seven chapters, style and content of this volume vary. What may interest one reader may be a subject for skipping by another, and it is unlikely that any one will find all subjects of equal merit. Topics range from early calculation through a variety of calculating machinery, including analogue computers, to relay and electronic calculators. Information given about work on computing equipment in Germany during World War II will be new to most readers. It offers some contrast with the British code-breaking computer, Colossus, built about the same time.

Occasional algebraic formulae or smidgions of calculus are to be found here, especially in the section on the difference engine and the chapter on analogue computers. Few users of digital computers feel comfortable with the concepts of analogue computing, perhaps because no two analogue computers ever seem to be alike.

The inclusion of a chapter on punched-card machinery might seem to be a diversion, and yet this technology played a significant part in paving the way for digital computers as the manufacturing companies involved formed the nuclei of the emergent computer industry. Quaint-looking punched-card readers were modernized by having their wrought-iron legs hastily covered up by metal boxes and

were adapted as data-input devices. Tabulators were modified to become the high-speed printers that computers needed if they were to be of value to the commercial user. But there was a long interval before computers (which were first used for scientific work) could take over the jobs done by punched-card machinery, and there was a further period when both types of equipment were used side-by-side and even offered in competition in the one bid. It is interesting to realize that punched-cards were used for scientific calculations despite their obvious bias towards mundane statistical and commercial applications.

Scientific and technological history is often presented as a string of success stories. This example of the genre is a salutary reminder that for every one success there were many failures. Most of the equipment described in this book was never finished or, if it was, never worked with any consistency. The authors conclude that, despite some similarities in general concepts, early computer equipment has contributed little to present designs.

Progress in computing since this history ends has been rapid. The book contains a photograph, courtesy of the Smithsonian Institute, of a slide-rule. For those of us who still own such an object, not yet in a museum, the caption is a measure of that progress and, perhaps, our own advancing years. □

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Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein

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The three-dimensional structure of the amino-terminal 44K ATPase fragment of the 70K bovine heat-shock cognate protein has been solved to a resolution of 2.2 Å. The ATPase fragment has two structural lobes with a deep cleft between them; ATP binds at the base of the cleft. Surprisingly, the nucleotide-binding 'core' of the ATPase fragment has a tertiary structure similar to that of hexokinase, although the remainder of the structures of the two proteins are completely dissimilar, suggesting that both the phosphotransferase mechanism and the substrate-induced conformational change intrinsic to the hexokinases may be used by the 70K heat shock-related proteins.

It has recently become apparent that there is a class of cellular proteins, commonly referred to as 'chaperones', whose proposed roles, broadly defined, include facilitating the correct assembly or disassembly of some oligomeric protein complexes and participating in the transmembrane targeting of certain proteins¹. Included in the ATP-dependent chaperones are the 70K heat-shock proteins (HSP70s) and HSP70-related proteins (relative molecular masses ~70,000 (70K)). Representatives of this group have been found in bacteria (for example the *Escherichia coli* dnaK protein)², chloroplasts^{3,4} and mitochondria⁵⁻⁷. Several other members are present in eukaryotic cells, including (1) strictly inducible representatives whose expression is induced by heat shock or other forms of cell stress², (2) constitutive representatives (denoted HSCs for heat shock cognates) which are present in the cell cytoplasm under normal conditions⁸, and (3) a representative in the endoplasmic reticulum, variously referred to as the glucose-regulated protein or BiP (for immunoglobulin heavy chain-binding protein)⁹. Although the biochemical functions of the HSP70-related proteins are not, in general, well delineated, the family is highly conserved, with, for example, the bacterial dnaK protein and eukaryotic HSP70-related proteins sharing ~48% amino-acid sequence identity². In all cases examined, the proteins are ATPases with K_m values for ATP ~10⁻⁶ M (ref. 10).

For the constitutive HSC70 proteins, there is evidence suggesting a role in disassembly of clathrin cages^{11,12}, as well as participation in post-translational transmembrane targeting of proteins to cellular organelles^{13,14}; one HSC70 protein is identical to β -internexin, a microtubule-associated protein¹⁵. The BiP proteins associate with subunits of oligomeric proteins in the endoplasmic reticulum, such as immunoglobulin heavy chains¹⁶ or protomers of the influenza haemagglutinin trimer^{17,18}. BiP may therefore have a crucial role in inhibiting the incorrect assembly of oligomeric proteins in the endoplasmic reticulum. No specific activities or associations have been identified for the inducible HSP70 proteins.

Proteolytic digestion of the bovine HSC70 protein (the clathrin uncoating ATPase) yields a 44K amino-terminal fragment which retains ATPase activity but is devoid of clathrin binding

activity¹⁹, demonstrating a segregation of the ATPase activity and the clathrin binding activity into separate functional domains. The high sequence conservation of the HSP70-related proteins suggests that the presence of two separate functional domains—a more highly conserved N-terminal 44K ATPase domain and a more variable C-terminal substrate recognition domain—will be common to members of the family.

Little information is available on the structure or ATPase mechanism of the HSP70-related proteins, or of other chaperone proteins beyond the recent report of the structure of the *E. coli* PapD protein²⁰. We have previously reported the isolation and crystallization of the 44K ATPase fragment of the bovine HSC70 protein²¹; here we report the three-dimensional X-ray crystallographic structure of the fragment and discuss its implications.

Structure determination

Crystals of the ATPase fragment of the bovine HSC70 protein were grown from polyethylene glycol in the presence of 1 mM Mg²⁺-ADP as previously described²¹. They are orthorhombic, space group P2₁2₁2₁, with cell parameters $a = 145.3$ Å, $b = 65.0$ Å, $c = 46.9$ Å.

Data were collected using both a diffractometer and a multiwire area detector. Collection was to 6.0 Å resolution on native crystals and several prospective heavy atom derivatives with a Picker four circle diffractometer equipped with a sealed tube X-ray source, using omega step scans. Gaussian profiles were fitted to the raw intensity data after background subtraction; transmission and decay corrections were computed and applied as described elsewhere^{22,23}. Additional data were collected with a multiwire area detector of the Xenotronics design equipped with a rotating anode X-ray source; integrated intensities were extracted from raw data frames using standard programs with local modifications^{24,25}.

Heavy atom sites for several isomorphous heavy atom derivatives were solved from difference Pattersons and difference Fouriers; heavy atom sites and occupancies were refined, and Blow-Crick 'best' phases were computed to 2.9 Å resolution by standard methods²⁶. Multiple isomorphous replacement (MIR) statistics are given in Table 1; the MIR phases, with overall figure of merit of 0.64, yielded an electron density map which, despite being noisy, had many interpretable features. The overall interpretability of the map was improved with solvent-flattening, using the method of automatic boundary definition of Wang²⁷; a solvent fraction of 0.50, computed from estimated protein volume and unit cell volume, was used.

Much of the overall backbone of the molecule could be traced in the solvent-flattened map. Model building was initiated with the modelling program FRODO²⁸ (Rice version 6.1) on an Evans and Sutherland PS 300 graphics system. At this stage, many of the connections between elements of well defined secondary structure (α -helices, β -strands) were vague; the amino-acid sequence of the ATPase fragment could not be positioned in unambiguous register in the fragmentary model. Consequently, residues whose side chains best fitted the apparent density were used. The initial model resulting from the first cycle of building consisted of 13 segments of polypeptide with a total of 295 amino-acid residues (as compared with 382 residues in the final model).

The initial model was refined by simulated annealing with native data to 2.5 Å, using the program package XPLOR²⁹. The

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TABLE 1 Heavy-atom derivative and refinement statistics

	Heavy-atom sites (No.)	Resolution (Å)	R_{sym}		Resolution limits (Å)								Overall
					30.0–13.8	13.8–9.0	9.0–6.7	6.7–5.3	5.3–4.4	4.4–3.8	3.8–3.3	3.3–2.9	
PTED	2	3.2	0.050	$f_{r.m.s.}/E_{r.m.s.}$	0.33	0.72	1.25	1.15	0.76	0.65	0.58	0.50	0.77*
ETHG	1	2.9	0.061	$f_{r.m.s.}/E_{r.m.s.}$	1.19	1.34	1.70	1.53	1.14	0.91	0.78	0.66	1.16
OSCL	3	2.9	0.058	$f_{r.m.s.}/E_{r.m.s.}$	1.28	1.16	1.23	1.30	0.90	0.77	0.68	0.65	1.00
PTD2	3	4.6	0.028	$f_{r.m.s.}/E_{r.m.s.}$	1.23	1.71	2.02	1.85	1.31	0	0	0	1.64†
HGOA	3	2.9	0.080	$f_{r.m.s.}/E_{r.m.s.}$	1.59	1.82	1.95	1.67	1.36	1.12	1.03	0.95	1.44
TRHG	1	2.9	0.054	$f_{r.m.s.}/E_{r.m.s.}$	0.90	1.07	1.35	1.27	0.96	0.80	0.71	0.72	0.97
NATI		2.2	0.078										
				Reflections (No.)	114	288	532	879	1,298	1,752	2,163	2,474	9,500
				Figure of merit	0.74	0.85	0.84	0.82	0.75	0.63	0.57	0.51	0.64

Derivative soaking conditions: PTED, saturated platinum ethylenediamine, overnight; ETHG, 10 mM ethyl mercury phosphate, overnight; OSCL, 1/10 saturated Na_2OsCl_6 , overnight; PTD2, saturated platinum ethylenediamine, overnight; HGOA, 2 mM $\text{Hg}(\text{CH}_3\text{COO})_2$, overnight; TRHG, 0.12 mM $(\text{CH}_3\text{COOH})_3\text{CCN}$, overnight; NATI, native data.

$$R_{\text{sym}} = \frac{\sum |I_i - \langle I \rangle|}{\sum \langle I \rangle} \quad f_{r.m.s.} = \left\{ \frac{\sum f_i^2}{n} \right\}^{1/2} \quad E_{r.m.s.} = \left\{ \frac{\sum (F_{\text{PH}} - F_p + f_i)^2}{n} \right\}^{1/2}$$

where I = diffraction intensity, f_i = calculated heavy-atom structure factor amplitude, F_p = structure factor amplitude of native crystals, and F_{PH} = structure factor amplitude of derivative crystals. Summations are over all reflections, n .

* Overall to 3.2 Å.

† Overall to 4.6 Å.

model resulting from the first cycle of refinement had an R -factor (as defined in Table 2) of 0.350. Phase combination between model and solvent-flattened MIR phases was effected as previously described^{30,31}; new maps to 2.5 Å resolution were computed. Many of the polypeptide connections could then be traced unambiguously, and much of the sequence (C.D-F. and D.B.M., manuscript in preparation; the amino-acid sequence in the ATPase fragment is identical to that of rat HSC70³²) could be placed. Six further cycles of model building, refinement, and phase combination were effected, using native data to 2.2 Å; the refinement statistics are summarized in Tables 2 and 3. A detailed discussion of the refinement is outside the scope of this article, and will be presented elsewhere. The current molecular model includes residues 3–384 of the amino-acid sequence; one or two residues at both the N and C termini could not be placed with confidence. A portion of a $(2F_o - F_c)$ map is shown in Fig. 1. In this article we discuss the overall tertiary structure of the HSC70 ATPase fragment and its implications.

Overall tertiary structure

The structure of the HSC70 ATPase fragment is shown in Fig. 2. A striking feature is the two lobes of approximately equal size, on the right and left in the view shown, with a deep cleft between them. Each lobe can be further subdivided into two separate topological domains. (The helices traversing the base of the cleft will, arguably, appear to be assigned to the incorrect structural domains; we have chosen this convention to clarify the discussion of the topology and its relation to other proteins.) In the view shown in Fig. 2, each lobe partitions into an upper and lower domain; the lower domains consist of five-strand β -sheets flanked by helices, whereas the two upper domains are composed of quite different combinations of helices and antiparallel β -strands. The overall topology of the protein is shown in Fig. 3a, with the separate domains in the schematic drawing having the same spatial relationship as the structural domains in Fig. 2, along with a convenient nomenclature for describing it: the right and left lobes are denoted I and II, respectively; the lower and upper domains are further designated as A and B, respectively. The domain boundaries can be taken to be: domain IA, residues 1–39 and 116–188; IB, 40–115; IIA, 189–228 and 307–end (presumably 385); IIB, 229–306.

It is notable that domains IA and IIA have identical topology, which can be described as $\beta\beta\beta \dots$ (B domain) $\dots \alpha\beta\alpha\beta\alpha$. Each of the domains begins with the central β -strand of the five-strand sheet, and runs directly into a second and third β -strand; the three strands are antiparallel. The polypeptide then proceeds to the IB and IIB domains, returns into an α -helix on the exterior of the molecule (distal to the cleft), and continues through a β -strand, exterior α -helix, β -strand, and a final α -helix that traverses the base of the cleft.

The similarity of domains IA and IIA extends beyond their topology to their tertiary structures. The domains are of roughly equal size (~110 residues for IA, ~130 for IIA), as are their individual structural elements, with the corresponding α -helices and β -strands (except for the two helices that traverse the base of the cleft) differing by at most two or three residues in length. The two domains, taken in their entirety, can be superimposed with only modest precision, (equivalent α -carbons align with a root-mean-square discrepancy of 3.7 Å), owing to such factors as differences in packing angles of α -helices on the β -sheets. However, the backbone atoms of the central three β -strands can be superimposed with a root-mean-square difference in atomic position of 1.7 Å. The rotation angle for superposition of the central three β -strands is 176°, demonstrating that the domains are related by a pseudodyad, or approximate twofold, axis. There is a weak sequence similarity between IA and IIA taken as a whole; when the structures are superimposed and the sequences of equivalent residues are compared, 12 are identical and another 15 are conservative substitutions³³ for the bovine HSC70 sequence, for an overall similarity of ~25%. A much stronger similarity is observed for the central three strands, including the turn connecting the two antiparallel strands: out of 22 residues, four are identical and another eight similar, for an overall similarity of 50%. For these three strands this level of similarity is maintained throughout the HSP70 family; a group of 14 diverse eukaryotic sequences (enumerated in the legend to Fig. 2) show 46% similarity (three residues identical, eight residues conservative substitutions out of 24) for the central three β -strands of IA and IIA. These data provoke the question of whether domains IA and IIA may have evolved from a common ancestral domain.

Similarity to hexokinase

When the trace of the polypeptide backbone was near completion, we looked for similarities between the HSC70 fragment and the structures of other proteins. Unexpectedly, part of the overall tertiary structure of the fragment is similar to the known structure of yeast hexokinase³⁴, despite the fact that members of the HSP70-related protein family and the hexokinase family have no significant global similarity in amino-acid sequence. A qualitative comparison to the hexokinase topology (Fig. 3b) illustrates the major points. Specifically, domains IA and IIA of the HSC70 fragment have essentially identical folding topology to segments of hexokinase, whereas domains IB and IIB show no similarity whatsoever to the corresponding regions in hexokinase. Domain IB of HSC70 fragment is essentially absent in hexokinase, whereas hexokinase has a pair of antiparallel β -strands following the fourth strand of the β -sheet that are absent in HSC70 fragment. Domain IIB of hexokinase is composed of an assembly of α -helices, in contrast to the make-up of the corresponding domain of HSC70 fragment.

TABLE 2 Summary of refinement statistics

Refinement cycle	Residues/peptide segments	r.m.s. bond deviation from ideality (Å)	r.m.s. angle deviation from ideality (degree)	R-factor
1	295 residues in 13 chains	0.030	5.5	0.350
2	352 residues in 4 chains	0.027	5.0	0.287
3	376 residues in 2 chains	0.021	4.0	0.245
4	383 residues in 1 chain + ADP	0.021	3.8	0.230
5	382 residues in 1 chain + ATP + 50 H ₂ O	0.020	3.7	0.218
6	382 residues in 1 chain + ATP + 55 H ₂ O	0.020	3.8	0.215
7	382 residues in 1 chain + ATP + 55 H ₂ O	0.018	3.6	0.205

Observed reflections with $F_o > 2\sigma(F_o)$ were used in refinement; cycle 1 included 12,773 reflections between 8.0 Å and 2.5 Å; cycles 2–7 included 16,790 reflections between 6.0 Å and 2.2 Å.

$$R\text{-factor} = \frac{\sum |F_o - F_c|}{\sum F_o}$$

where F_o = observed structure factor amplitude = $\langle I \rangle^{1/2}$, $\langle I \rangle$ = mean measured intensity; F_c = structure factor amplitude calculated from model; r.m.s., root mean square.

Further, hexokinase has a segment of ~80 residues before the beginning of domain 1A that is not present in the HSC70 fragment. Beginning at the N terminus of hexokinase, two β -strands and an α -helix are present which interact with lobe II of the enzyme before the peptide migrates to the beginning of domain 1A.

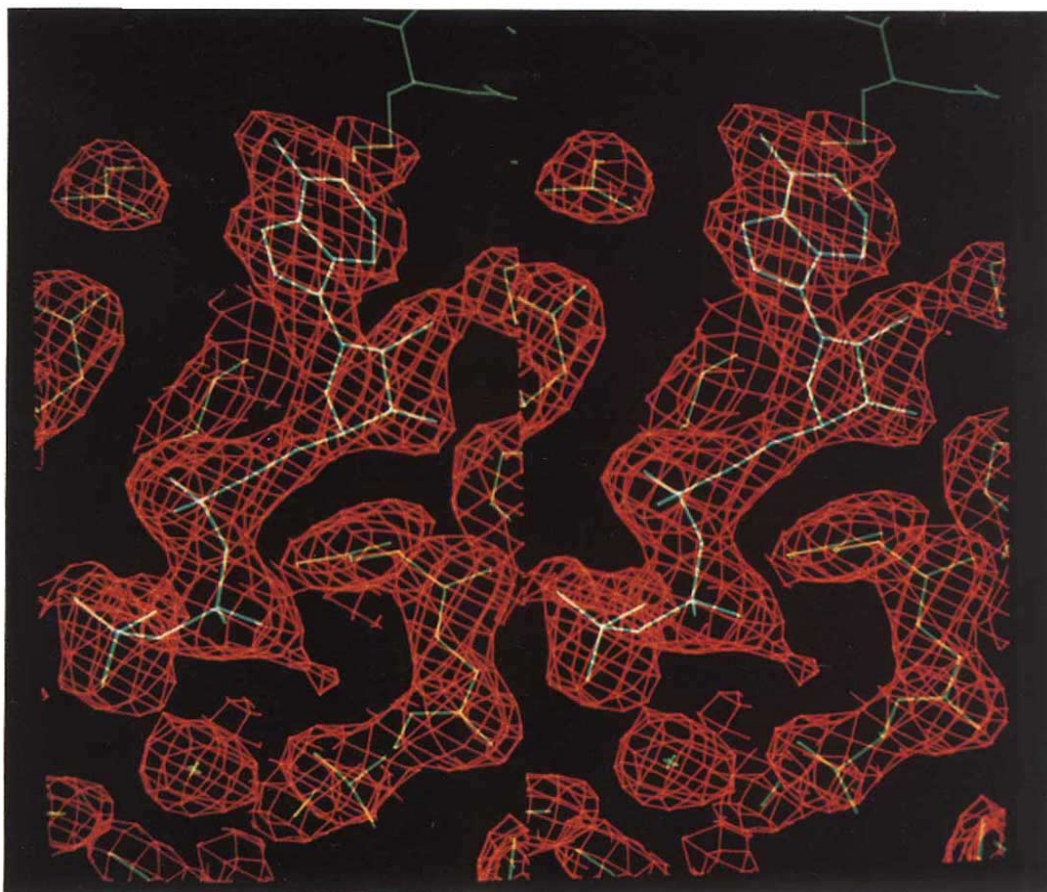
As the overall similarity in structure of the ATPase fragment of HSC70 to that of kinase suggests that the HSC70 protein might function as a kinase, we have looked for such an activity; however, efforts to demonstrate either phosphorylation of clathrin components or autophosphorylation during the uncoating reaction have yielded only negative results. A search for autophosphorylation of HSC70 in the presence of Ca^{2+} ATP, an activity which has been observed for the *E. coli* dnaK protein³⁵, also yielded negative results. However, these results do not, by themselves, preclude the possibility that reactions of the HSP70-related proteins with their substrates may involve (short-lived or long-lived) phosphorylated intermediates. It is notable, in

this context, that some dnaK[−] strains of *E. coli* show deficiency in phosphorylation of three proteins, including two transfer RNA synthetases³⁶, although direct phosphorylation of these proteins by dnaK *in vitro* has not yet been demonstrated.

Nucleotide-binding site

During model-building, electron density for a nucleotide revealed itself at the base of the cleft of the fragment. As the crystals were grown in the presence of 1 mM Mg^{2+} ADP, an ADP molecule was initially built into the density and refined, but difference Fouriers revealed a persistent density contiguous with the β -phosphate which could accommodate an additional phosphate, as well as a single isolated peak which might be a metal ion. The distance between the peaks of density for the β and terminal phosphates was too small to accommodate separate inorganic phosphate and ADP molecules; hence an ATP molecule was built into the model (Fig. 1). Thin-layer chromatography of redissolved crystals confirmed the identity

FIG. 1 Stereo view of a $2F_o - F_c$ electron density map (orange), computed using model phases and contoured at 1.5σ , with the molecular model (green). The bound ATP is illustrated.



of the nucleotide as ATP. It is probable that the observed nucleotide is residual ATP from chromatographic elution of the fragment from ATP-agarose (the final step of its purification) which is not hydrolysed under the crystallization conditions. Although the HSC70 fragment is active in ATP hydrolysis at pH 9.5 (the pH of crystallization) under low ionic strength conditions, it is inhibited by high concentrations of monovalent cation¹¹, and in particular, by 1M NaCl which is included in crystallization.

We have subsequently adapted crystals to low ionic strength conditions (20% polyethylene glycol, 50 mM buffer, 1 mM Mg^{2+} ADP, pH 9.5). Difference Fouriers on data collected to 2.2 Å resolution from these crystals revealed the bound nucleotide to be ADP under these conditions, a result that was corroborated by thin-layer chromatography of redissolved crys-

tals. Hence, at low ionic strength the bound ATP is hydrolysed to ADP in the crystals, demonstrating that the ATP is bound at an active site. The original mechanistic proposal of Rothman and coworkers for the clathrin uncoating reaction by the intact protein suggested two ATP binding sites, one of which is hydrolytic, and the other of which is 'catalytic' but does not hydrolyse ATP¹⁰; our results confirm the identity of the observed binding site as the hydrolytic site.

As can be seen in Fig. 1, the adenine ring is in the *anti* position relative to the sugar, and the ribose has a 2'-*endo* pucker. The β - and γ -phosphate conformation is distorted from what one would expect for a productive Mg^{2+} -ATP complex, judging from observations on other phosphotransferase enzymes³⁷. The observed conformation of the ATP, combined with the observation that the position of the nucleotide base and sugar does not

FIG. 2 *a*, Stereo view of the α -carbon backbone of the HSC70 ATPase fragment, along with the ATP molecule. The different colours correspond to the different structural domains: domain IA, green; IB, cyan; IIA, orange; IIB, magenta. *b*, Schematic drawing of the structure. Picture produced by a program written by A. M. Lesk and K. D. Hardman^{41,42}.

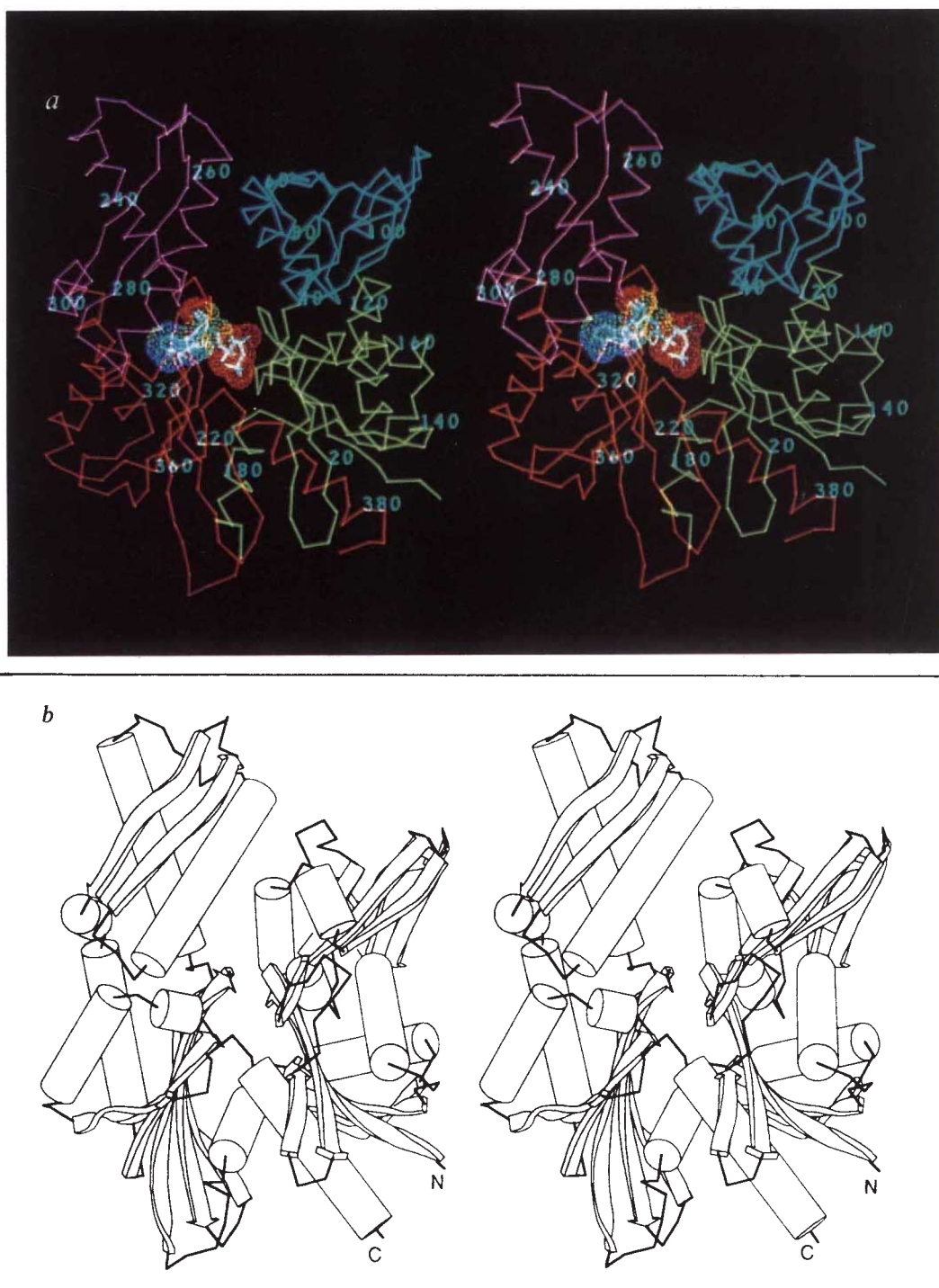


TABLE 3 Refinement statistics versus resolution for refinement cycle 7

Resolution range (Å)	No. of reflections with $F > 2\sigma(F)$	Completeness of data*	Mean $\langle F_{\text{nat}} \rangle$	R-factor
6.00–3.99	2,820	0.983	15.80	0.1642
3.99–3.34	2,765	0.991	12.20	0.1760
3.34–2.97	2,453	0.886	8.74	0.2183
2.97–2.73	2,215	0.806	7.23	0.2310
2.73–2.55	1,945	0.705	6.32	0.2430
2.55–2.41	1,760	0.648	5.56	0.2786
2.41–2.29	1,504	0.549	5.49	0.2672
2.29–2.20	1,328	0.491	6.05	0.2582
6.00–2.20	16,790	0.760	9.18	0.2050

* Ratio of number of reflections measured with $F > 2\sigma(F)$ to total number of reflections in resolution shell.

shift substantially after hydrolysis at low ionic strength in the crystal, suggests that inhibition of the ATPase activity at high ionic strength may be a result of distortion of the conformation and/or local environment of the terminal phosphates.

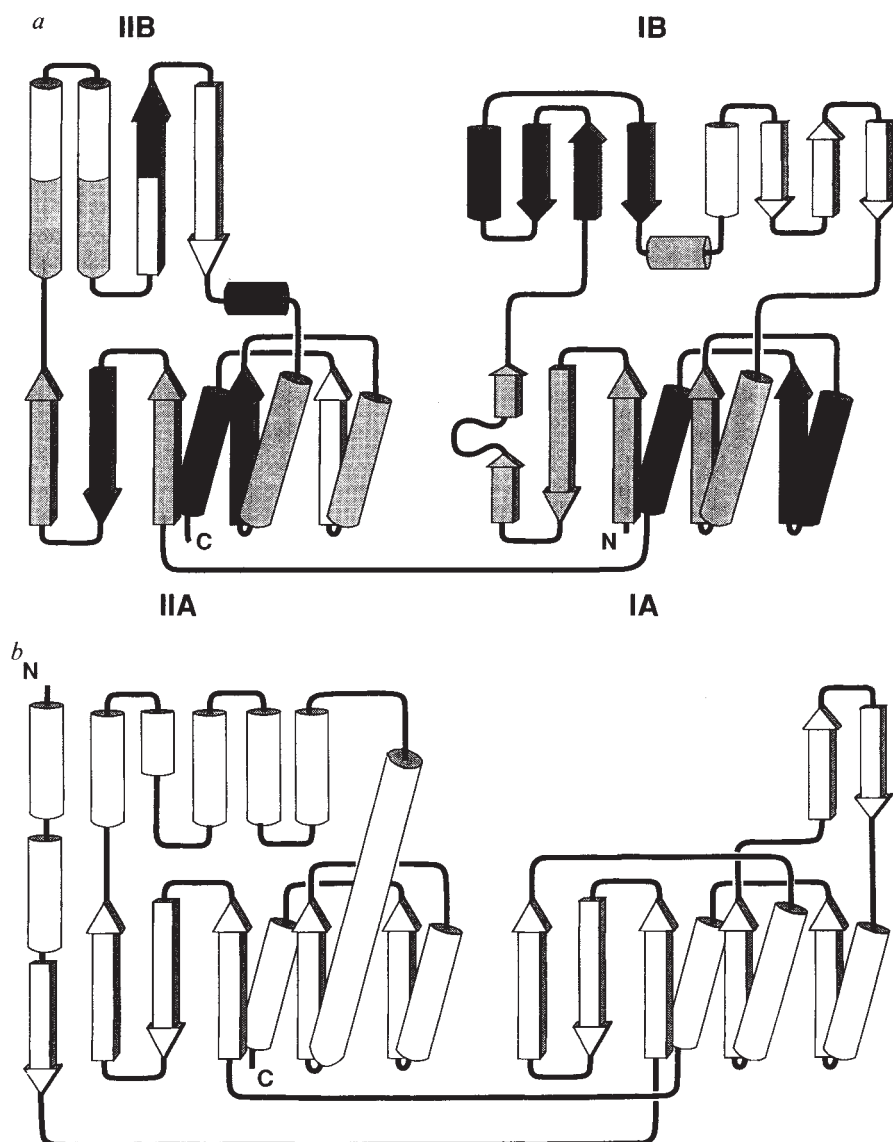
We are deferring a detailed discussion of the ATP-protein interactions until refinement and comparisons of differences of the ATP-bound and ADP-bound structures are completed. But our results so far suggest candidates for the catalytic proton

acceptor anticipated to be involved in the ATPase mechanism³⁷. One candidate is Glu 175; it is an equivalent position to the proposed catalytic base of hexokinase³⁸, Asp 211; further, it is strictly conserved in all known sequences of HSP70-related proteins. A second alternative, based on its proximity to the γ -phosphate of the ATP, is Asp 10; this is also strictly conserved among all HSP70-related proteins and in fact is part of a conserved N-terminal sequence: Gly-Ile-Asp-Leu-Gly-Thr-Thr. Despite the lack of global sequence similarity between hexokinases and the HSP70-related proteins, it is notable that the hexokinases have a similar sequence, Ala-(Ile/Leu)-Asp-Leu-Gly-Gly-(Thr/Ser), in the same position³⁹, raising the question of whether this particular aspartic acid has a crucial catalytic role in both systems.

Conserved versus variable regions

The HSP70-related proteins in general, and their ATPase domains in particular, are highly conserved in amino-acid sequence. Within this context, some regions of the ATPase domain are more highly conserved than others. A relatively rudimentary alignment and comparison of 14 eukaryotic sequences, in which an overall level of sequence conservation is computed for the peptides corresponding to each α -helix and β -strand, yields the result displayed in Fig. 3a: the most highly conserved elements are shaded dark grey; regions of

FIG. 3 a, Topology of the HSC70 ATPase fragment. α -helices (cylinders) and β -strands (arrows) are shaded according to the degree of amino-acid sequence conservation: Sequences of 14 representative eukaryotic HSP70-related proteins available in the Genbank sequence database were aligned by eye. For each α -helix and β -strand, a similarity parameter $P = (I + 0.5S)/T$ was computed, where I , number of positions where residue is identical for all sequences; S , number of positions where residues all fell into a single conservative substitution group (where conservative substitution group 1 is C; 2 is S, T, P, A, G; 3 is N, D, E, Q; 4 is H, R, K; 5 is M, I, L, V; and 6 is FYW (single-letter notation for amino acids))³³; T , total number of positions. Shading code: dark grey, $0.75 \leq P \leq 1.0$; light grey, $0.50 \leq P \leq 0.74$; unshaded, $P \leq 0.49$. The apparent relative levels of conservation are relatively insensitive to the specific form of the expression for P . b, Topology of hexokinase.



intermediate (moderate) conservation are light grey; the more variable regions are not shaded. It is clear that most of domains IA and IIA are moderately to highly conserved, as are the helical parts of IIB that are proximal to IIA. It is likely that conservation of these regions is mandated by the functional imperatives of the ATPase activity; this is the region of the protein that appears to interact directly with the nucleotide.

A region that is highly conserved, but which is remote from the putative ATP binding site and does not have any direct interaction with the nucleotide in our model, is the first part of domain IB, more specifically the initial $\beta\beta\alpha\beta$ of the domain. This region is on the same side of the molecule as the C terminus of the ATPase fragment (and hence the beginning of the substrate-binding domain). It is notable that the upper portion of the first β -strand of domain IIB, another highly conserved region, is also on this 'face' of the molecule. Additionally, the rather unusual protrusion which interrupts the third β -strand of domain IA, a feature whose length (eight residues) is constant and whose sequence is strongly homologous within the HSP70 protein family (for example, 75% sequence similarity between 14 eukaryotic sequences), also lies on this surface. These areas, taken together, present a highly conserved surface patch on the molecule. (In the view shown in Fig. 2, this patch is on the upper right front surface of the molecule, whereas the C terminus of the fragment is at the lower right corner.) If one assumes that specific contacts between the ATPase fragment and the substrate-binding fragment of the HSP70 proteins that are required for function are likely to be conserved, then it is reasonable to suggest that these regions form the surface of the ATPase fragment which interacts with the substrate-binding domain of the molecule.

Implications

The specific functions of members of the HSP70 protein family have not been unambiguously delineated; however, many of the diverse activities in which they have been implicated, such as ATP-dependent disassembly of clathrin cages by HSC70¹¹, and participation in assembly of oligomeric proteins in the endoplas-

mic reticulum by BiP (which is presumed to be ATP-dependent^{17,18}), require recognition of a specific substrate and suggest that a substantial conformational change must be transmitted from the protein to the target substrate. In the absence of a structure for the substrate recognition domain of the HSC70 protein, we cannot shed any light on the question of how it recognizes such a seemingly diverse ensemble of proteins as specific substrates. The structure of the ATPase fragment, however, and in particular the unanticipated observation that the ATP-binding core of the HSC70 fragment has an identical topology and similar tertiary structure to the ATP plus glucose binding core of hexokinase, gives some insight into how energy of hydrolysis of ATP may be transduced, through a substantial conformational change, to a substrate-HSC70 protein complex.

Steitz and colleagues have shown for hexokinase that substrates bind in a cleft between structural lobes of the protein, and that formation of the catalytically active ATP binding site is induced by binding of glucose (or other substrates), which results in a transition from an 'open' to a 'closed' substrate-binding cleft^{38,40}. The glucose-induced conformational change is substantial, resulting from a rotation of 12° of one lobe of the protein relative to the other. The similarity of the structure of the HSC70 ATPase fragment to that of hexokinase suggests that a conformational change of similar magnitude might be possible on binding or hydrolysis of ATP by the HSP70-related proteins. It is well established that the HSC70 protein by itself binds ATP tightly but does not hydrolyse it appreciably until it binds a target substrate such as clathrin. It is probable that under appropriate conditions, substrate-induced ATP hydrolysis can trigger the ATPase domain to shift from a 'closed' to an 'open' conformation, with this conformational change being transmitted to the substrate recognition domain, thereby transducing free energy of hydrolysis of a nucleoside triphosphate to a substantial conformational change in a target substrate.

Note added in proof: After submission of this manuscript, we were informed by Dr Ken Holmes that the folding topology of actin is nearly identical to that of the HSC70 ATPase fragment. □

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Viral escape by selection of cytotoxic T cell-resistant virus variants *in vivo*

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Viruses persist in an immune population, as in the case of influenza, or in an individual, as postulated for human immunodeficiency virus, when they are able to escape existent neutralizing antibody responses by changing their antigens. It is now shown that viruses can in principle escape the immunosurveillance of virus-specific cytotoxic T cells by mutations that alter the relevant T-cell epitope.

THE adaptive immune response usually protects vertebrate hosts against virus infections either through virus-specific T cells or neutralizing antibody responses¹. Some viruses may persist despite an immune response by hiding in privileged sites¹⁻³ or by changing patterns of cytopathogenicity⁴⁻⁷, growth kinetics or tissue tropisms⁴⁻⁹. Other viruses escape by down-modulating viral antigen¹⁰, antigen-presenting major histocompatibility complex (MHC) gene products^{11,12}, by inducing immunosuppressive factors¹³⁻¹⁷ or by changing neutralizing surface determinants¹⁸⁻²⁶. This has been analysed *in vitro* with single neutralizing monoclonal antibodies but has also been shown at the population level for influenza^{18,22-24} and has been postulated for individuals in the case of human immunodeficiency type-1 (HIV-1) virus^{27,28}. Here we demonstrate a novel mechanism: lymphocytic choriomeningitis virus (LCMV), a negative-stranded RNA virus with a high natural mutation rate²⁹⁻³², may escape MHC-dependent immune surveillance in an individual by *in vivo* selection of virus mutants that are resistant to recognition by cytotoxic T cells. Although this demonstration depends on an acute infection and is in transgenic mice in which the repertoire of selecting T cells is very limited, similar stepwise selections may play a part in establishment of persistent slow or chronic virus infections in individuals.

Mice transgenic for a virus-specific TCR

LCMV, a non-cytopathic RNA virus²⁹⁻³² induces in the mouse, its natural host, a protective cytotoxic T-cell response resulting in both virus elimination³³⁻³⁵ and T-cell mediated immunopathology³⁶⁻³⁸. This cytotoxic T-cell immune response to LCMV has been analysed here in two lines of transgenic mice (305 and 327) that express the T-cell antigen receptor (TCR) α ($V\alpha 2$) and β ($V\beta 8.1$) chains derived from the cytotoxic T-cell line P14 that is LCMV glycoprotein-specific (amino acids 32-42)^{39,40} and H-2D^b restricted. Cytofluorometric analysis with $V\alpha 2$ - and $V\beta 8$ -specific monoclonal antibodies revealed that the transgenic TCR was expressed on 75-90% of peripheral T cells in the transgenic lines 305 and 327 (Fig. 1). Correspondingly, T-cell immunocompetence in these mice is reduced roughly threefold when assayed by alloreactivity or MHC-restricted T-cell responses (not shown).

Virus elimination

TCR transgenic mice eliminated the WE strain of LCMV (LCMV-WE) in the spleen very rapidly after i.v. infection with

a dose of 10^2 p.f.u. (Fig. 2, upper left) but not when infected with high doses (10^6 p.f.u.) of the LCMV-WE assayed 8 days or 15 days later (Fig. 2, upper middle and right panels). The decline in virus titres noticeable between day 8 and day 15 probably reflects virus elimination by endogenous TCR-expressing T cells present at a low frequency. Similar results were obtained when virus titres were determined in liver, kidney and thymus (data not shown). This contrasts with nontransgenic control mice which efficiently eliminated LCMV by day 8 or 15. As spleen cells from both transgenic and nontransgenic mice were highly cytolytic against target cells infected with LCMV (Fig. 2 lower panel), which usually reflects efficient antiviral protection^{33-35,41}, these findings suggested that the LCMV variants had been selected in TCR transgenic mice to escape immune recognition by T cells expressing transgenic receptor.

Virus variants

LCMV virus isolated from spleen on days 2, 4, 8 and 15 after infection from transgenic and nontransgenic mice was analysed either directly (Fig. 3a) or after plaque purification (Fig. 3b) with cytotoxic T lymphocytes (CTL) *in vitro* with the following

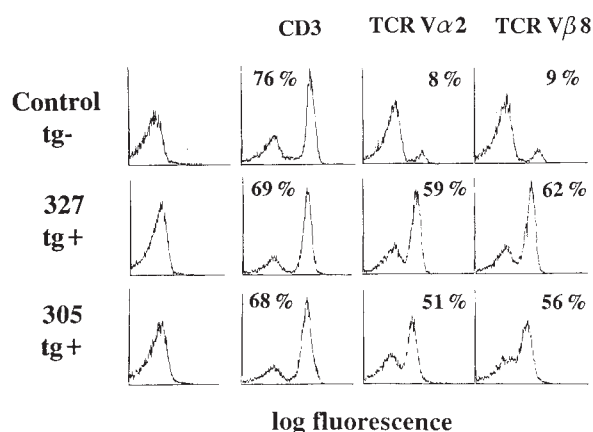


FIG. 1 Cell-surface expression of transgenic TCR chains. Lymph node cells derived from transgene-negative and from TCR-transgenic mice (line 327 and 305) were stained with monoclonal antibodies specific for CD3, transgenic α chain ($V\alpha 2$) and transgenic β chain ($V\beta 8$). Percentages of positively stained cells are indicated.

METHODS. The generation of the TCR-transgenic mice has been described previously^{53,54}. In brief, the P14 TCR α and β chain constructs were co-injected into (C57BL/6 $\times$ DBA/2)F2 generation fertilized eggs. The founder animals 305 and 327 bearing 10-20 copies of both α and β transgenes integrated at the same chromosome were back-crossed to C57BL/6 (H-2^b) mice. The examined transgene heterozygous offspring were identified by immunofluorescence of peripheral blood lymphocytes with TCR $V\beta$ -specific antibodies. For cytofluorometric analysis, single cell suspensions from mesenteric lymph nodes were incubated first with monoclonal antibody KT3 (anti-CD3)⁵⁵, B20.1 (anti- $V\alpha 2$; B. Malissen, manuscript in preparation) and KJ16 (anti- $V\beta 8.1$ and 8.2)⁵⁶. The cells were washed and stained with goat anti-rat IgG-FITC (TAGO, Burlingame, California) and analysed by flow cytometry on a Epics profile Analyzer using logarithmic scales.

results: target cells infected with LCMV variants isolated as bulk viruses on day 8 and day 15 from transgenic mice were not recognized in a ⁵¹Cr release assay by activated cytotoxic T cells expressing transgenic TCR but were readily recognized by the cytotoxic T-cell clone 50.1 specific for LCMV-WE glycoprotein (aa275–288)^{39,42} or by day-8 LCMV immune T cells from normal C57BL/6 mice. Correspondingly, of 54 cloned virus isolates derived from three separate spleens of transgenic mice line 305 infected with 10⁶ p.f.u. LCMV-WE 8 or 15 days previously, only two were recognized by the transgenic TCR *in vitro* (Fig. 3c). As a control, target cells infected with original LCMV-WE or with bulk or cloned virus isolates from nontransgenic mice infected for 8 days were lysed by transgenic T cells. LCMV variants seemed to appear in substantial numbers only after day 4 in high dose (10⁶ p.f.u.)-infected transgenic mice, as bulk virus isolated on day 2 and day 4, but not on day 8 from transgenic spleens was recognized by the transgenic TCR.

In contrast to 10² p.f.u. of wild-type LCMV-WE, the same dose of three randomly selected triple plaque-purified LCMV variants (12.1, 8.6 and 8.7) were not cleared in transgenics by day 4 (Fig. 4a). These variants were derived from the spleens of two transgenic mice (line 305) 8 days after high dose infection (10⁶ p.f.u.). For further experiments, variant 8.7 was chosen. Variant 8.7 did not activate transgenic T-cells *in vitro* or *in vivo* (Fig. 4b, left panel). However, control C57BL/6 mice immunized with this LCMV variant induced cytotoxic T-cell responses active against target cells infected with the original LCMV-WE or against variant infected target cells (Fig. 4b, right panel). C57BL/6 mice immunized with LCMV-WE generate cytotoxic T-cells specific for two LCMV glycoprotein epitopes (amino acids (aa) 32–42 and 275–288 (ref. 43); Fig. 4c). It is noteworthy that the same mice infected with the LCMV variant 8.7 showed a reduced cytotoxic response against the aa32–42 epitope when

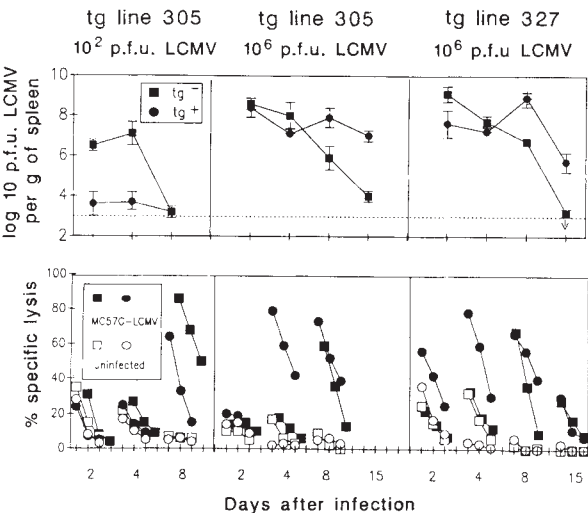


FIG. 2 LCMV infection in TCR-transgenic mice. Transgenic mice (●) and transgene-negative littermates (○) of the indicated lines were infected with LCMV-WE. Virus titres in the spleen (top panel) and the virus-specific cytolytic activities of splenocytes (bottom panel) were determined at the indicated days after infection. The lytic activities were determined in a ⁵¹Cr release assay on MC57G (H-2^b) target cells infected (●, ■) and noninfected (○, □) with LCMV-WE.

METHODS. Mice were infected by intravenous injection of 10² or 10⁶ p.f.u. of the WE strain of LCM virus (originally obtained from Dr F. Lehmann-Grube, Hamburg, FRG) which was plaque-purified three times and produced in L929 fibroblast cells. The quantification of infectious virus in spleens has been described previously⁵⁷. In brief, organs were weighed and homogenized, cleared of debris and titrated on L929 cell cultures. Titres are expressed as p.f.u. per g of spleen, on the basis of the analysis of at least three mice ± s.e. per group. The lytic activities of the spleen cells were determined in a 4–5-h ⁵¹Cr release assay at an effector/target ratio of 30:1, 10:1 and 3:1 on day 2, 4, 8 and 15 after infection as described³⁹.

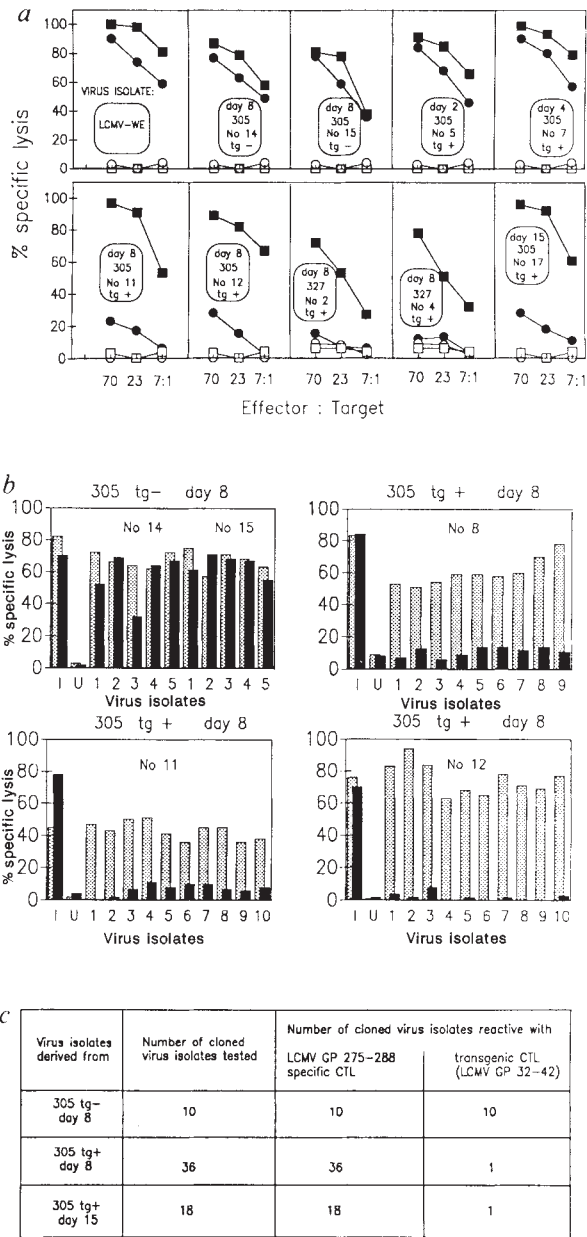


FIG. 3 LCMV isolates derived from TCR transgenic mice infected with 10⁶ p.f.u. of LCMV-WE are not recognized by the transgenic TCR. **a**, MC57G (H-2^b) target cells were left uninfected (○, □) or were infected with LCMV-WE or with bulk virus isolates from the indicated individual mice and tested in a ⁵¹Cr release assay with cytotoxic T cell clone (50.1) specific for the LCMV WE glycoprotein (aa275–288)^{39,42} (■) and with transgenic effector cells (●) specific for LCMV-WE glycoprotein (aa32–42). **b**, MC57G infected with individual single plaque-purified isolates from mice numbers 14, 15, 8, 11 and 12 were tested with LCMV glycoprotein aa278–286 (dotted bar) and aa32–42 (solid bars) specific effectors cells. The numbers below the figures indicate individual plaque-purified isolates. LCMV-WE-infected (I) and uninfected (U) target cells were included in each assay. The effector to target ratio was 10:1 for T-cell lines and 30:1 for effector spleen cells. **c**, Summary of the reactivity assays of plaque-purified isolates from the indicated origins towards the transgenic TCR. A cloned virus isolate was judged reactive when virus-specific cell-mediated lysis was above 15% in the ⁵¹Cr release assay.

METHODS. MC57G (H-2^b) cells were infected with titrated bulk virus spleen homogenates from infected mice or with one time plaque-purified isolates for 2 days with a multiplicity of infection of 0.01–0.1 and used as target cells. Effector cells were: a cytotoxic T cell clone (50.1) specific for LCMV glycoprotein aa275–288^{39,42} and transgenic T cells activated day 4 *in vivo* (10⁶ p.f.u.) or cytotoxic T-cell lines from 327 TCR-transgenic mice specific for LCMV glycoprotein aa32–42. The virus was plaque-purified on L929 cells and stocks were grown in the same cells as described elsewhere⁵⁸.

compared with mice infected with LCMV-WE (Fig. 4c, right panel). The cytotoxic response against the aa275–288 epitope was comparable in mice infected with LCMV-WE and in mice infected with LCMV variant 8.7 (Fig. 4c, middle panel). identical results were obtained with another independent LCMV variant (12.1) (data not shown). These findings suggested that the isolated LCMV variants express an altered glycoprotein aa32–42 epitope.

Point mutations within the T-cell epitope

We have defined aa32–42 on the LCMV-WE glycoprotein as the antigen epitope for the transgenic TCR by examining overlapping peptides (data not shown). We therefore determined the corresponding nucleotide sequence of a fragment of the LCMV glycoprotein gene (residues 60–530) comprising the transgenic T-cell epitope (residues 171–203) from three cloned virus variants (12.1, 8.6, 8.7), from one bulk virus variant isolate (12.0) and from the wild-type WE strain used. The four sequenced virus variants contained single nucleotide changes at position 177, 180 and 184 when compared with wild-type WE virus^{44,45} (Fig. 5a). For each cloned virus variant (12.1, 8.6, 8.7) four cDNA clones containing 470 bp of the LCMV glycoprotein gene derived from two independent polymerase chain reaction (PCR) amplifications were sequenced with the same result. Additional quality controls for the PCR methodology are the sequence comparison of 440 base pairs of the LCMV glycoprotein gene fragment outside the epitope region, which did not show any nucleotide difference between wild-type WE and variants (not shown). Sequence analysis of seven independently cloned LCMV glycoprotein gene fragments from the bulk virus variant 12.0 revealed identical results, suggesting a predominance of one virus variant type. The deduced amino-acid changes in the LCMV variants correspond to aa 34, 35 and 36 (Ala 34 → Thr, Val 35 → Leu, Tyr 36 → Phe, Tyr 36 → Cys) within the transgenic T-cell epitope. To examine whether these changes affected binding of the transgenic TCR receptor directly, or abolished presentation of the peptide by H-2D^b, mutant peptides from two virus variants (12.0 and 8.7) were synthesized and tested in a ⁵¹Cr release assay. Target cells coated with the wild-type LCMV-WE peptide aa32–42 or with the variant 12.0 mutant peptide (Tyr 36 → Phe) were readily lysed by CTL against LCMV from C57BL/6 mice, whereas targets presenting 8.7 mutant peptide (Val 35 → Leu) were lysed to a lesser degree (Fig. 5b, upper panel). By contrast, CTLs from transgenic mice infected with 10⁶ p.f.u. of LCMV-WE lysed targets presenting LCMV-WE wild-type peptide, but not targets coated with mutant peptide (Fig. 5b, lower panel). These results show that LCMV variants were not recognized by the transgenic TCR because of mutation in the relevant T-cell epitope and that the mutated peptides could still be recognized by T-cells immune to LCMV-WE from C57BL/6 mice. This indicated that the amino-acid substitution at positions 35 and 36 interfered with transgenic TCR recognition rather than with binding to MHC D^b molecules.

Discussion

Cytotoxic T-cell responses are able, during a primary infection *in vivo*, rapidly to select mutant virus that escapes the dominant T-cell recognition and as a consequence is no longer efficiently eliminated in these transgenic mice. Such variants were readily detected in TCR transgenic mice because T-cell recognition is mainly directed against one viral epitope with high frequency and therefore potentially protective T-cell responses against other LCMV epitopes are either not generated at all or appear relatively late in the TCR transgenic mice. LCMV variant selection is probably rare in the normal mouse; usually the different MHC class I alleles expressed in one individual, presenting different viral antigen fragments, should prevent the unlikely occurrence of the many mutations that would permit immunological escape. However, variant selection by T cells may play a part in normal individuals in a multistep process and/or under

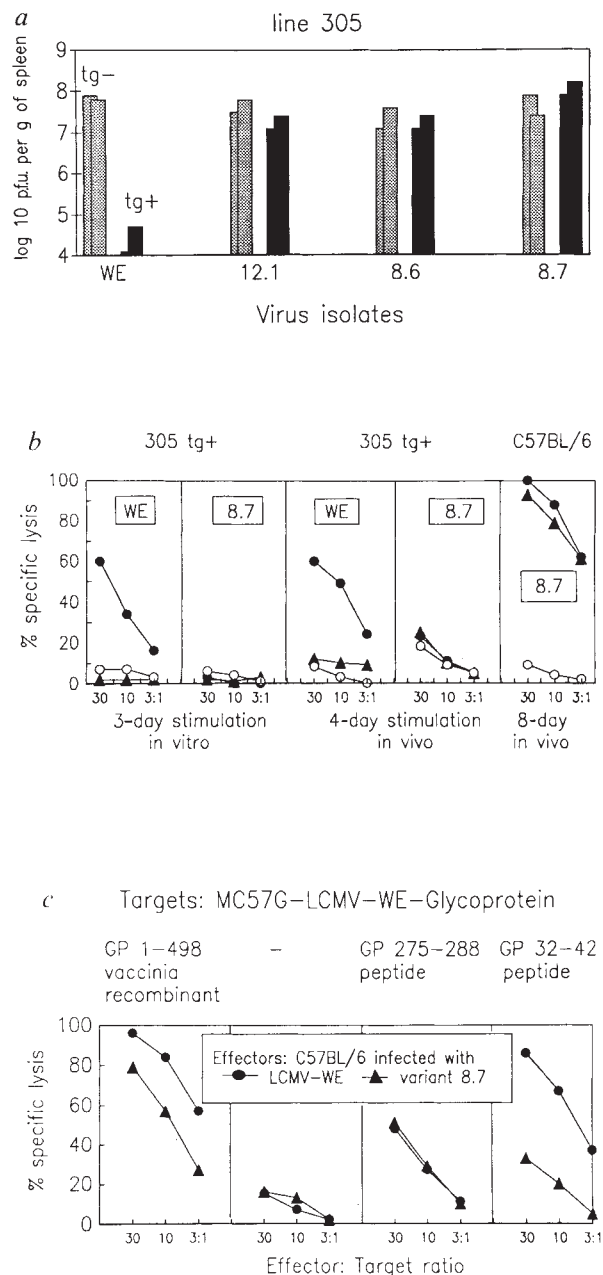


FIG. 4 LCMV variants were not eliminated in transgenic mice and did not activate transgenic T cells. **a**, Transgene-negative littermates (dotted bars) and transgenic mice (solid bars) were infected i.v. with 10² p.f.u. LCMV-WE or with the same dose of the indicated triple plaque-purified LCMV variants. Virus titres of the spleens were determined after 4 days. Individual bars represent separate mice. **b**, Spleen cells of transgenic mice line 305 were activated *in vitro* (left panel) or *in vivo* (middle panel) with LCMV-WE or LCMV variant 8.7 and tested on MC57G-LCMV-WE (●), MC57G-LCMV-8.7 (▲) and uninfected target cells (○). As a control, immune spleen cells from C57BL/6 mice infected *in vivo* with LCMV variant 8.7 were included in the assay (right panel). **c**, C57BL/6 mice were infected with 10² p.f.u. of LCMV-WE (●) or LCMV-8.7 (▲) and after 8 days the spleen cells were assayed against the indicated target cells.

METHODS. Infectious virus was quantified as described in Fig. 2. Transgenic T cells were activated *in vitro* by culturing spleen cells ($5 \times 10^6 \text{ ml}^{-1}$) with irradiated, LCMV-infected macrophages ($4 \times 10^5 \text{ ml}^{-1}$) for 3 days. To stimulate transgenic T cells *in vivo*, mice were injected i.v. on day -4 with 10⁶ p.f.u. LCMV-WE. MC57G target cells were infected with vaccinia recombinant virus (5 p.f.u. per cell) expressing the LCMV-WE glycoprotein⁵⁹ or loaded with the indicated peptides at 100 μM 2 h before the assay. The peptides IKAVYNFATCG (single-letter amino-acid code; aa32–42) and SSGVENPGGYCLTK (aa275–288) of LCMV-WE glycoprotein were synthesized by solid-phase method and purchased from Neosystem Laboratoire (Strasbourg, France).

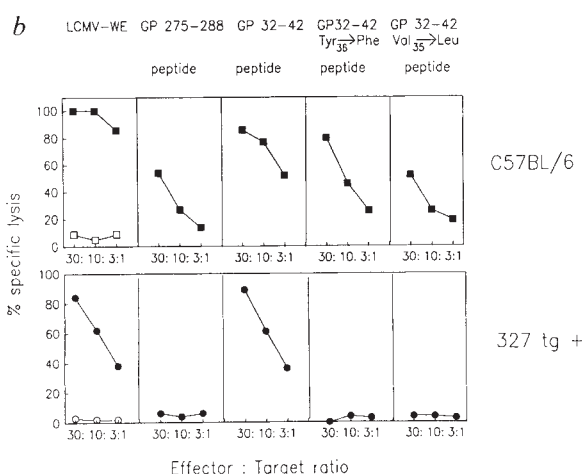
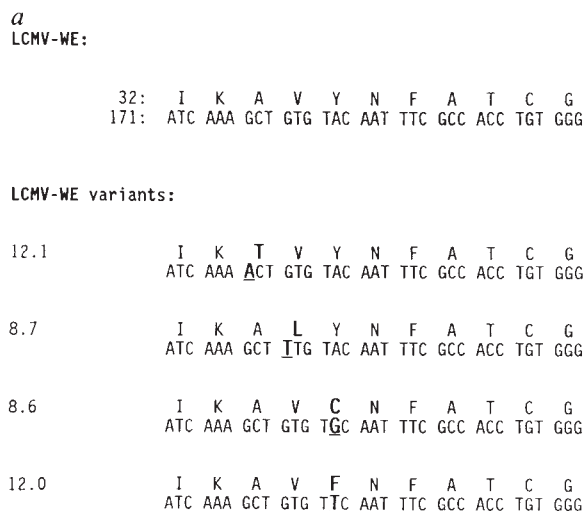


FIG. 5 LCMV variants contain point mutations within the transgenic T-cell epitope. **a**, Nucleotide and deduced amino-acid sequence of the transgenic T-cell epitope region on the glycoprotein of LCMV-WE and its variants. The nucleotide sequence is given as the viral-sense DNA⁴⁴. **b**, Target cells coated with mutant peptides 8.7 and 12.0 were not recognized by transgenic effector T cells. MC57G target cells coated with the indicated peptides were tested in a Cr⁵¹ release assay with LCMV immune spleen cells from C57BL/6 (■) or from 327 transgenic mice (●). MC57G (□, ○) target cells infected and uninfected with LCMV-WE were included as a control.

METHODS. LCMV virus was purified from supernatant of infected L929 cells and viral RNA was extracted as described elsewhere⁴⁵. Viral RNA (1 µg) was then used as a template for complementary DNA synthesis with reverse transcriptase (M-MuLV, Pharmacia) using the manufacturers conditions and a specific primer (5'-GGTACCGATAGCTTGTTGGCTGCACC). One-fiftieth of

the cDNA mixture was amplified by PCR using *Taq* DNA polymerase (Boehringer Mannheim) in 100 µl standard buffer and a second specific primer (5'-GAATTCTATCCAGTAAAGGATGG). This primer was designed to hybridize with the 5' end of LCMV glycoprotein gene and to contain an *Eco*RI restriction site. PCR was run for 42 cycles using a thermal cycler (Perkin Elmer Cetus). Denaturation was for 1 min at 94 °C, annealing for 2 min at 42 °C and extension for 3 min at 72 °C. The amplified 703-bp fragment was digested with *Eco*RI and the resulting 473-bp fragment was cloned into pUC-18. Positive colonies were sequenced by the dideoxy chain termination method (Sequenase, USB). In the epitope region both strands were sequenced. To avoid PCR artefacts, for each virus variant four cDNA clones derived from two independent PCR amplifications were sequenced, with the same result. The mutant peptides (IKAVNFATCG and IKALYNFATCG) were used at 100 µM.

conditions of for example, high doses of infectious virus, non-specific immunosuppression alone or together with either inefficient viral clearance by neutralizing antibodies or oligo-monospecific T-cell responses against one viral epitope, or with both.

These experiments document that, like antibody-driven selection, cytotoxic T-cell responses may also efficiently select virus mutants in a given individual. Antibody-driven selection of mutant virus has been demonstrated *in vitro* with single neutralizing antibodies; however it cannot be induced with two or more distinct monoclonal antibodies at the same time, but only if they are used in sequence²². Presumably as a result of this multistep selection, the virus may spread through a species population. In contrast, cytotoxic T-cell-dependent selection is relevant to the individual only. Because cytotoxic T cells recognize virus as peptide fragments of various viral gene products in a strictly class I MHC-dependent fashion^{46,47}, virus can survive immune selection only in this individual (or individuals with the same major transplantation antigens).

It is probably because of the high natural mutation rate of LCMV and the virtually exclusive importance^{1,33-35,41} of cytotoxic T cells rather than antibodies in early virus control that virus mutant selection *in vivo* could be found during a primary LCMV infection. As infection by HIV, like LCMV, may not induce protective antibodies very efficiently, partly because of antibody-driven selection of variants^{27,28}, it is possible that time-dependent cytotoxic T cell-driven selection may also play a part in persistence of virus in slow or chronic virus infections in humans, such as AIDS and possibly also in measles SSPE (subacute sclerosing panencephalitis)⁴⁸. Similarly, one may speculate that Epstein-Barr virus-induced⁴⁹ as well as other lymphohaemopoietic⁵⁰ or other tumour cells⁵¹, or parasites such as malaria⁵² may escape protective cytotoxic T-cell responses by mutating the relevant MHC-dependent epitope. □

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LETTERS TO NATURE

Generation of large-scale cosmological structures by gravitational clustering

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A VARIETY of observational evidence attests to the presence of large voids, filaments and sheets in the distribution of galaxies on large cosmological scales. Similar structures have been produced by numerical simulation of the gravitational evolution of a distribution of initial density fluctuations, but there has been controversy over which initial conditions are needed to produce the desired result: the interpretation of simulations is generally hampered by their discreteness, low resolution both spatially and in mass, and poor statistics. Here we present the results of a set of high-resolution two-dimensional simulations starting from gaussian initial conditions^{1,2}. Such a distribution, in which the phases of the Fourier components of the density fluctuations are distributed randomly, is the 'least special' of initial states, and is believed to have a physical origin in quantum noise in the early Universe. By systematically comparing results from simulations in which the power spectra varied but the initial phases were kept the same, we argue that filamentary structure is a general result of nonlinear gravitational evolution, and requires less power on large scales than has often been believed.

High-resolution two-dimensional simulations, despite evident restrictions, have an important advantage in that, for the same effort, we can achieve a resolution of $R^{3/2}$ for a two-dimensional simulation, where R is the resolution of a three-dimensional simulation. Typically $R_{3D} \approx 10^2$, and therefore R_{2D} can easily be $\sim 10^3$. Our two-dimensional models are equivalent to a cross-section of a Friedmann universe (with a cosmological density parameter $\Omega = 1$) that is homogeneous perpendicular to the plane of the calculation.

To differentiate between features owing to a preferred scale in the primordial spectrum or to its nonlinear evolution, we studied some pure power-law initial conditions. We chose power laws $P \propto k^n$, where n is 2 (Q series), 0 (J series) or -2 (N series). These two-dimensional simulations are analogues of three-dimensional simulations with $P \propto k^{n-1}$, in the sense that integrals over the power spectrum (like the density and potential) have the same dependence on k . Thus the Q series is analogous to the flat Harrison-Zel'dovich spectrum, the J series is approximately an analogue of the observed power spectrum of the present Universe, and the N series is the limiting case of cold dark matter on very small length scales and has a weak dependence of density contrast on length scale. Some studies of pure power laws in three dimensions with less dynamic range have been described³, but did not deal with the same issues we consider here. Low-resolution two-dimensional studies using the adhesion approximation have given preliminary indications that agree with our conclusions on filamentarity⁴. Recent lower-

resolution adhesion and particle simulations also agree with some of our results, but that study does not include the variety of cut-offs and power spectra that we considered.

To test systematically the origins of structure we did simulations for each of the Q, J and N series using sharp cut-offs of the initial power spectra at $k = 4, 32$ and 256 . The latter cut-off corresponds to the Nyquist frequency and is the maximum possible in this simulation. It is the best possible approximation to a pure power law given the size of the arrays we are using (512^2 particles on a 512^2 mesh in a particle-mesh code¹). For $k > k_c$ we set to zero the amplitude of all Fourier coefficients in the initial conditions.

To make direct comparison possible we used the same phases for the corresponding components k_{ij} in each simulation. Thus the only difference between, for example, the J4, J32 and J256 series is the presence of extra initial power on small length scales. We can therefore isolate and study the effects of power on small length scales and of the spectral index n .

By using a large dynamic range we avoided the possibility of filaments arising from sparse sampling in k space. Also, in an experiment not shown here, we began with a Poisson particle distribution and observed that similar structures to those seen in the J256 simulation evolved. The particle lattice is therefore not the source of filaments. We will discuss these results in further detail elsewhere.

We also had to decide which 'moment' in each simulation to compare with the others. We chose to compare them at the same k_{NL} (NL, nonlinear) where

$$\left\langle \left(\frac{\delta \rho}{\rho} \right)^2 \right\rangle_{k_{NL}} \equiv \int_0^{k_{NL}} |\delta_k|^2 d^2k = 1 \quad (1)$$

where ρ is the density and the δ_k are the result of linear extrapolation from the initial conditions. Here we discuss the relation of k_{NL} to the formation of voids and filaments.

Figure 1 shows the nine simulations, each in comoving coordinates, taken at the moment when $k_{NL} = 4$, where $k = 1$ is the fundamental mode of the simulations.

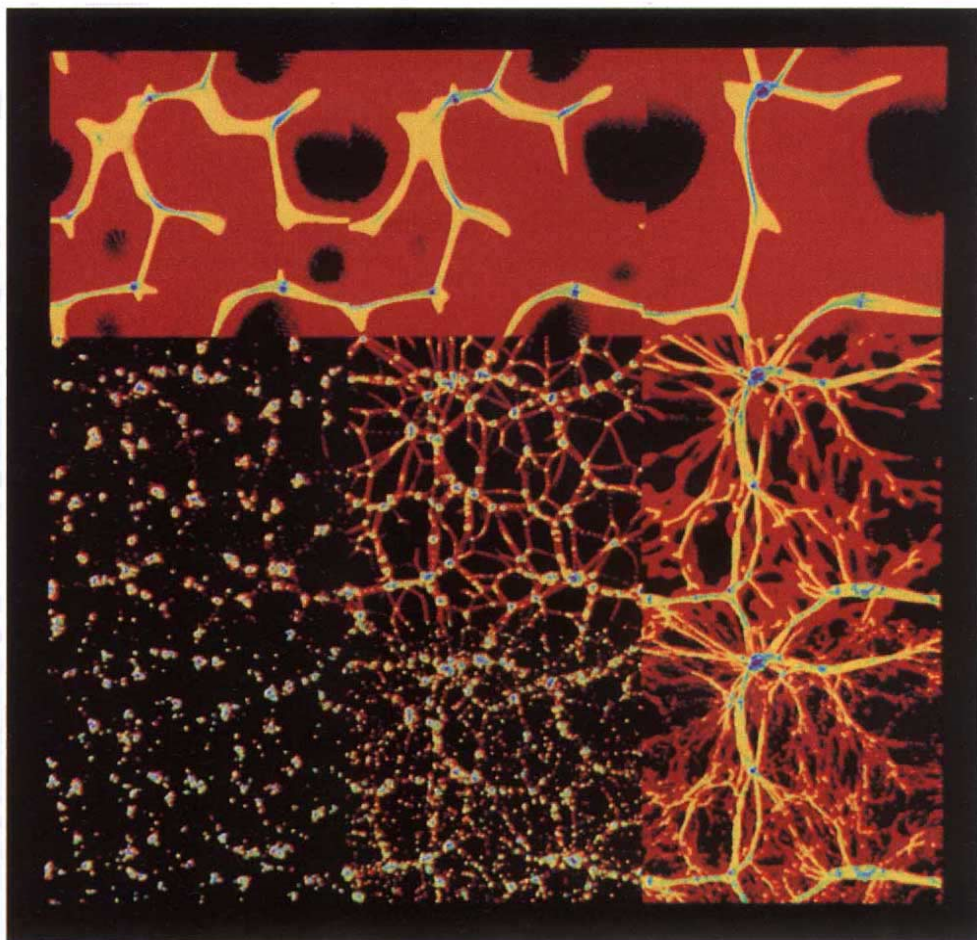
All J series ($n = 0$, which we believe is similar to $n = -1$ in three dimensions) show similar structure (Fig. 1). In other words, when $k_{NL} = 4$ the aligned arrangements of lumps in J32 and J256 series follow closely the pattern in the 'classic pancake' model J4. We call these 'second-generation' pancakes in the hierarchical models (that is, the pancakes are much larger than any initial coherence length), blurring the distinction between what used to be called 'adiabatic' and 'isothermal' theories. As the simulation evolves, these structures evolve to larger scales. The aligned structures in J256 appear much larger than where the two-point correlation function crosses unity $R_1 \approx 0.03L$ or even zero $R_0 \approx 0.06L$, L being the side of the square simulation region.

Except for differences of small amplitude, we find only four different power spectra for these nine evolved distributions, despite the fact that they can all be distinguished visually. Although none of them (and, of course, none of the two-point correlations) are simple power laws, we find that power spectra are easier to analyse, and acquire at late times a roughly triangular form, in a logarithmic plot (Fig. 2).

Our results confirm several earlier results. A very prominent filament-void structure arises in all models with cut-offs when

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FIG. 1 Composite of our nine simulations: The top row has the cutoff $k_c=4$ in the initial conditions, the middle at $k_c=32$ and the bottom had power at frequencies up to Nyquist frequency (256). The left column is the Q series, the centre is the J series and the right is the N series. The colour code is as follows. Below $\frac{1}{3}$ the mean mass density is black. Regions near the mean are red, and violet corresponds to a mass density of 10^3 . The increments are logarithmic.



$k_{NL} \approx k_{cut-off}$. The N series all show this structure, indicating that a feature or cut-off in the initial spectrum is not needed if n is negative and large enough. Our nine models confirm the widely held belief that in the nonlinear regime two different power spectra (or two-point correlation functions) imply different patterns, but various patterns can have nearly identical spectra.

From our simulations we conclude, and we believe that this applies to the three-dimensional case as well, that non-gaussian initial conditions or a bump in the primordial power spectrum are not needed for the formation of a void-filament structure with a typical scale about 10 times larger than the correlation length for an index $n < 0$ in two-dimensions or $n < -1$ in three dimensions. Because the cold-dark-matter power spectrum⁷ goes

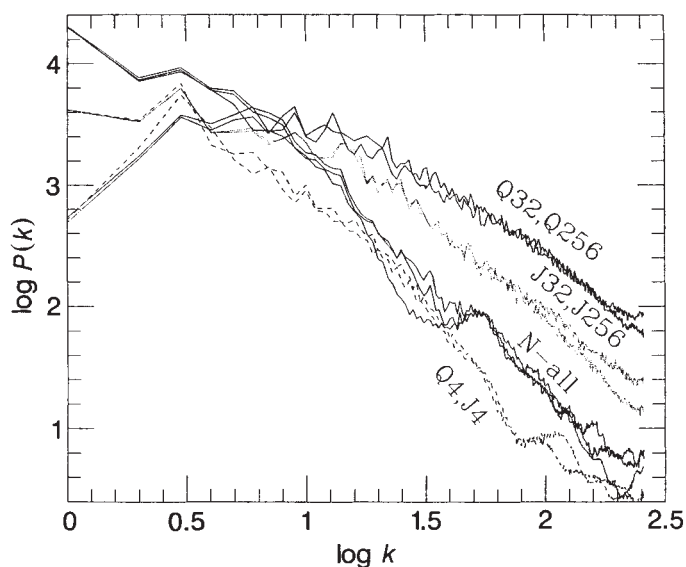


FIG. 2 Power spectra of the distributions shown in Fig. 1.

through $n = -1$ at a wavelength of ~ 50 Mpc for a Hubble constant of $50 \text{ km s}^{-1} \text{ Mpc}^{-1}$, structures of this size are expected. Occasionally they can be a few times larger. We see by comparison of spectra for various cut-offs that the Zel'dovich 'pancake' approximation⁶, usually applied in detail only to truncated power spectra or to very-low-amplitude initial conditions, may provide a good qualitative description of the gravitational collapse on some length scales even when there is significant

nonlinear substructure on smaller scales provided that the spectrum is steep enough.

It remains to be seen whether gaussian initial conditions, which can easily produce the appropriate morphology for large-scale structure, can do so for a power spectrum that does not violate observational constraints on the microwave background. $\square$

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Contamination of the Th II line and the age of the Galaxy

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THE age of the Galaxy may be estimated from observations of the ratio of stellar abundances of thorium, which has only one long-lived isotope with a half-life comparable to the suspected age of the Galaxy, and neodymium, a stable element. The Th/Nd abundance ratio in a sample of G-dwarf stars of different ages was derived by Butcher¹ from the intensities of one Th II and one Nd II absorption line, and indicated a rather young galactic age of 9.6 Gyr. But the Th II line is blended with a Co I line. Here we determine the transition probability of the Co I line by combining radiative lifetime and branching-ratio measurements. We show that the Co I contribution cannot be neglected in deriving Th/Nd ratios. By comparing our results with predictions based on models of galactic chemical evolution, we suggest a revised age of the Galaxy of 15–20 Gyr.

The Co I line at 401.9126 nm lies in near perfect coincidence with the Th II line at 401.9129 nm (ref. 2) used to determine Th abundances¹. Because the Co I line is more sensitive to temperature, its contribution to the absorption at 401.913 nm will

very from star to star. The contribution of the Co I line can be separated if its oscillator strength is known accurately. As the calculation and the one unpublished measurement cited by Holweger² differ by a factor of ten, we have remeasured it by combining measurements of the radiative lifetime and the branching ratios.

Accurate (5%) radiative lifetimes can be measured using time-resolved laser-induced fluorescence on atomic and ionic beams³. This technique is essentially free from systematic error and is applicable to all metallic atoms and ions using an atomic/ionic beam source based on a hollow-cathode discharge³. We measured the branching ratios using the 1.0-m Fourier transform spectrometer (FTS) at the US National Solar Observatory⁴. The FTS produces very high resolution (Doppler-limited) spectra from the ultraviolet to the infrared with excellent ($1 \text{ part in } 10^7$) absolute wavenumber accuracy. A Co hollow-cathode lamp containing Ar was used in the branching ratio work, the argon emission lines being used to calibrate the relative efficiency of the FTS as a function of wavenumber⁵. The precision and accuracy of this approach for determining oscillator strengths is well established⁶.

We measured the lifetime of the Co I $w^4D_{3/2}$ level using laser excitation at 259.169, 261.786 and 439.189 nm. The agreement between the measurements for the three lines indicates that the lines have been correctly identified in our experiment, were correctly classified in the literature, and are unblended. We found the lifetime of the $w^4D_{3/2}$ level to be $21 \pm 1 \text{ ns}$.

To determine oscillator strengths by combining lifetime and branching-ratio measurements, it is necessary to have a complete

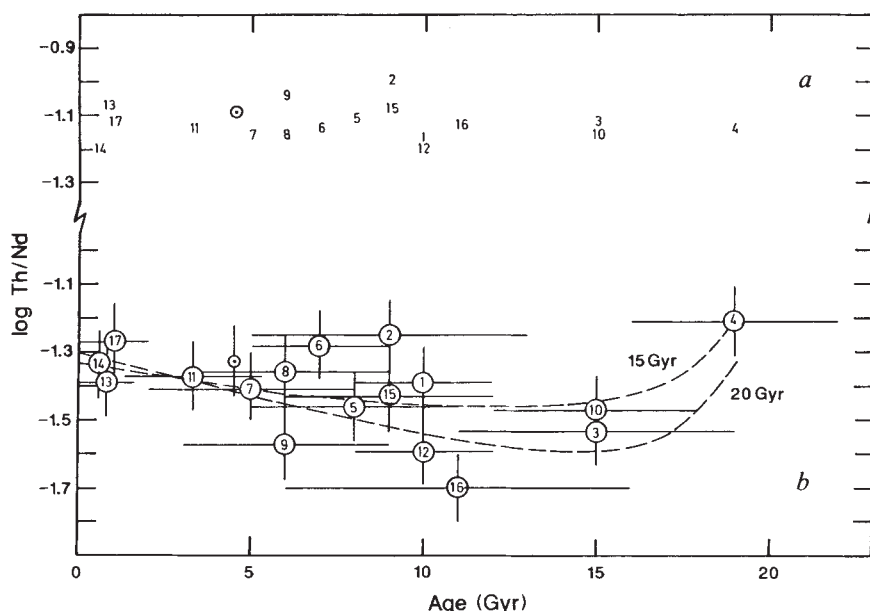


FIG. 1 Logarithms of Th/Nd as a function of age of G-dwarf stars studied by Butcher¹. The numbering of stars corresponds to those in Tables 1 and 3 of ref. 1. The results in *a* do not include the effect of the contamination of the Th II line by the Co I line, whereas the results in *b* do. Uncertainties on the stellar ages are from ref. 1, and the uncertainty of ± 0.10 on the abundances is our estimate. Dashed lines show the predictions by Mathews and Schramm¹⁸. Very recently, Arnould and Takahashi (personal communication) have developed a new model for the galactic evolution of the *r*- and *s*-processes that is in very good agreement with the results of ref. 18. In this model it is assumed that a tiny amount of *r*-process elements are already present at very early times and that the *r*/*s* ratio remained constant during the evolution of the Galaxy. This model reproduces both the general behaviour of the observed Th/Nd ratio and the available Eu/Ba ratios.

TABLE 1 Transitions from the Co I $w^4D_{3/2}$ level at 43,263.57 cm^{-1}

Wavenumber (cm^{-1})	Wavelength in air (nm)	Lower level	Branching fraction (%)	$\log(gf)$
41,856.73	238.8374	$a^4F_{5/2}$	9.1 ± 0.7	-1.83 ± 0.04
38,573.39	259.1686	$b^4F_{5/2}$	17.5 ± 0.8	-1.47 ± 0.03
38,187.74	261.7860	$b^4F_{3/2}$	9.8 ± 0.4	-1.72 ± 0.03
34,802.76	287.2479	$a^2F_{5/2}$	4.4 ± 0.3	-1.98 ± 0.03
29,227.29	342.0479	$a^4F_{3/2}$	8.5 ± 0.4	-1.55 ± 0.03
28,864.29	346.3497	$a^4P_{1/2}$	3.2 ± 0.2	-1.96 ± 0.04
28,079.53	356.0296	$b^4P_{5/2}$	7.5 ± 0.8	-1.57 ± 0.05
27,489.53	363.6712	$b^4P_{3/2}$	15.2 ± 0.8	-1.24 ± 0.03
27,067.89	369.3363	$b^4P_{1/2}$	7.9 ± 0.8	-1.51 ± 0.05
26,792.97	373.1261	$a^2D_{3/2}$	2.7 ± 0.2	-1.97 ± 0.04
26,485.41	377.4591	$a^2D_{5/2}$	8.0 ± 0.4	-1.49 ± 0.03
24,874.00	401.9126	$a^2P_{3/2}$	1.2 ± 0.1	-2.27 ± 0.04
22,762.86	439.1887	$b^2P_{3/2}$	3.0 ± 0.2	-1.78 ± 0.03
20,111.00	497.1016	$b^2D_{3/2}$	0.75 ± 0.08	-2.28 ± 0.05
15,766.51	634.0805	$c^2D_{5/2}$	1.0 ± 0.1	-1.92 ± 0.04

or nearly complete set of emission branching ratios. Our measurements of such a set, called branching fractions, are given in Table 1. We made a careful search for weak transitions obeying the $|\Delta J| \leq 1$ and parity selection rules between the $w^4D_{3/2}$ and all known Co I levels. There is a blended line at 241.165 nm from the $w^4D_{3/2}$ to a $4^4F_{3/2}$ level. Although we were unable to make an accurate measurement of the branching ratio of this blended line, a laser-induced fluorescence measurement indicates that the branching ratio of this line is less than or equal to that of the line at 238.8374 nm. We performed this measurement by spectrally resolving the enhanced emission from the $w^4D_{3/2}$ level after laser excitation of the level. This result is an upper limit because the measurement, unlike our lifetime measurements, was made in a hollow-cathode discharge where collisional mixing can be important. If the 241.165-nm line is as strong as the 238.8374-nm line, which is unlikely, then all other $\log(gf)$ values in Table 1 would be lowered by 0.04. New theoretical oscillator strengths (R. L. Kurucz, personal communication) show that the intensity of the 241.165-nm line is only 13% of the intensity of the 238.8374-nm line, indicating that the branching fraction of the 241.165-nm line is 1.2% and therefore negligible. Our measured oscillator strength, $\log(gf)$, of -2.27 ± 0.04 for the Co I line at 401.9126 nm indicates that it should not be neglected in the abundance determinations using syntheses of solar or stellar spectra at 401.9 nm.

To evaluate the effect of the Co I line, we calculated the Co I contribution to the total Th II + Co I feature for the solar centre as well as for the solar disk. Using a solar abundance, $A(\text{Co})$, of 4.92 (ref. 7) (where $A(\text{Co}) = \log[N(\text{Co})/N(\text{H})] + 12$), $\log(gf) = -2.27$ (this work), and the photospheric model of Holweger and Müller⁸, we calculate equivalent widths W_λ of 1.85 mÅ for the solar centre and 2.35 mÅ for the solar disk. The total equivalent widths are $W_\lambda = 6.0 \pm 0.2$ mÅ for the solar centre as remeasured on the Jungfraujoch solar atlas of Delbouille *et al.*⁹, and 7.4 mÅ for the solar disk¹, showing that Co I contributes 31% to the observed line and is therefore not negligible. The contributions of Th II are 4.15 mÅ for the solar centre and 5.05 mÅ for the solar disk.

From these equivalent widths, we calculated the solar abundance of Th. The transition probability of the Th II line has been derived recently by Simonsen *et al.*¹⁰ from accurate lifetime measurements. Their $\log(gf)$ value, -0.10 ± 0.04 , when corrected to be consistent with their lifetime of 23.0 ± 0.7 ns and their branching fraction of 85%, is -0.27 ± 0.04 . We found the same thorium abundance, $A(\text{Th}) = 0.23 \pm 0.08$, from the solar-centre and solar-disk observations, within the uncertainties of the various measurements. This result was derived using partition functions for Th II that were directly calculated from the recent work on the atomic energy levels¹¹. At 5,000 K, the new values are $u(\text{Th I}) = 27$ and $u(\text{Th II}) = 37$. This result for Th II is much larger than Irwin's value¹², 9.2, and is in rough agreement with the value of Corliss and Bozman¹³.

Our solar abundance of thorium is $\sim 40\%$ larger than the most recent meteoritic result⁷, 0.08 ± 0.02 , a difference that is difficult to explain. The use of a single Th II line to determine an abundance requires that the equivalent width, the contribution of all other lines, and the Th atomic data be accurately known. Except for the Co I line, we could not find any other line that might be blended with the Th II line. The meteoritic abundance should also be accurate because Th is a refractory element, tied to U and Pb by isotope systematics, and is unlikely to have been selectively depleted in meteorites (E. Anders, personal communication).

The contribution of the Co I line to the total Th II + Co I feature has been calculated for each of the stars in ref. 1, including the Sun, using theoretical atmospheres interpolated from the models of Kurucz¹⁴ and Peytremann¹⁵. These model atmospheres correspond to the characteristic $T(\text{eff})$, $\log g$ and metallicities given by Butcher. For each star the Co abundance adopted is derived from the solar Co abundance and the metallicity. Our results show that the Co I contribution is not at all negligible: we found values ranging from 19% to 86% of the equivalent widths observed by Butcher for the Th II + Co I line, with very large variations from one star to the other, depending on the effective temperature and metallicity. The Th II contributions are therefore smaller, sometimes much smaller than the values quoted in ref. 1. We also derived the neodymium abundances from the Nd II line observed by Butcher at 401.8823 nm. The oscillator strength of this line has been accurately measured by Ward *et al.*¹⁶, $\log(gf) = -0.89$. In the Sun, the abundance based on this line is in perfect agreement with the results derived from other Nd II lines. Our results for the Nd abundance in Butcher's G-dwarf stars are well correlated with the metallicities, $(A(\text{Nd}, \text{star}) - A(\text{Nd}, \text{Sun})) - [\text{Fe}/\text{H}] = 0.014 \pm 0.011$. The metallicity, $[\text{Fe}/\text{H}]$, is defined as the difference between a mean heavy-element abundance in the star and the Sun.

Our Th/Nd ratios (the solar value has also been derived with a theoretical solar model in order to compare results) are shown in Fig. 1 where we have plotted the logarithm of this ratio as a function of the ages given in Butcher¹. The values obtained without taking the Co I contribution into account (Fig. 1a) show a small dispersion and do not indicate any variation of the Th/Nd ratio with stellar age. Our results obtained using stellar model atmospheres are very similar to the results Butcher obtained using one-layer models. Although the dispersion of the results increases when the Co I contribution is taken into account (Fig. 1b), they now suggest a variation of the Th/Nd ratio with stellar age. If the only result for a very old star (19 Gyr) is reliable, the Th/Nd ratio may first decrease with stellar age, followed by an increase for very old stars. The increase in dispersion of the results in Fig. 1b may be due to inaccuracies in the effective temperatures of the stars; the Co I contribution is much more temperature sensitive than the Th II or Nd II lines.

The different models for and problems in determining the galactic age using the Th–Nd chronometer have been reviewed recently¹⁷. There are a wide range of models and they cannot all be included here. Some of the models that include the possible differences between the s- and r- process histories during the chemical evolution of the Galaxy might be able to reproduce the observed evolution of the Th/Nd ratio for galactic ages of the order of 15–20 Gyr. Ages in this range are much higher than the rather short value, 9.6 Gyr, obtained by Butcher¹.

The predictions of such a model are shown in Fig. 1b (dashed line); they show the same general behaviour as the observational results obtained when the contribution of Co I is taken into account but disagree with them if it is not. Such a model would lead to a large variation of the Eu/Ba ratio with age, however, which is not confirmed by the observations on stars with rather high metallicities¹⁹, but which is observed when a much larger range of metallicities is considered²⁰.

The Eu/Ba ratio is derived by Butcher²¹ from the Eu II line at 412.972 nm and from the Ba II line at 413.066 nm. In G-dwarf

stars these lines are much stronger than the Th II and Nd II lines. The Eu II line shows hyperfine structure and the Ba II line is blended with a Ce II line, however, and one might therefore question these Eu/Ba ratios.

The new results do not resolve the many questions about the Th–Nd chronometer because the inclusion of the Co I contamination increases the scatter in the Th/Nd ratio. Furthermore, there are large differences between prediction of recent models of the galactic chemical evolution. Our results do show that the Co I contamination must be included in analysing the Th data, however, and that it affects the plot of Th/Nd ratio against age, providing a better constraint for models of the chemical evolution of the Galaxy. □

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MWC560—a unique astrophysical object

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DESPITE its interesting behaviour^{1,2}, the object MWC560 has been little studied since its discovery 50 years ago during the Mount Wilson Observatory H α emission-line surveys³. To rectify this situation we have made systematic spectral and photometric observations of this star. Spectra of the hydrogen absorption Balmer lines show remarkable variability: they indicate that some components have radial velocities as large as $-6,000 \text{ km s}^{-1}$, and the profiles are complex, showing pronounced variability on timescales of a few days. Photometric observations show that the V-band brightness of the star has risen by about three magnitudes over the past 20 years; during our own observations, this brightness was seen to change by a few tenths of a magnitude. We speculate that MWC560 may be a binary system containing a red giant and a compact companion—possibly a white dwarf—and exhibiting jet-like ejections along the line of sight⁴.

In 1973 Sanduleak and Stephenson¹ observed strong blue-shifted absorption and variable emission lines, and TiO bands

TABLE 1 UBv observations of MWC560

Date	Julian date (HJD2447...)	V band (mag)	B–V colour (mag)	U–B colour (mag)	Observatory
February 1990					
24	947.482	9.65	0.28	–0.48	R
25	948.383	9.83	0.31	–0.41	B
26	949.368	9.93	0.29	–0.51	B
26	949.384	9.83	0.27	–0.53	R
28	951.357	9.80	0.26	–0.56	B
March 1990					
3	954.268	9.66	0.34	–0.54	B
4	955.387	9.54	0.31	–0.50	B
5	956.349	9.62	0.30	–0.46	B
5	956.354	9.65	0.28	–0.51	R
14	965.276	9.82	0.33	–0.44	R
14	965.353	9.84	0.35	–0.46	R

Average standard deviation is ± 0.02 mag. HJD is the heliocentric Julian date.

* R, National Astronomical Observatory Rozhen; B, Belogradchik Observatory.

in the visible and infrared regions of objective-prism spectra of MWC560, and assigned it a spectral type of M4ep. Later, Bond and co-workers³ noted the presence of broad ($\sim 3,000 \text{ km s}^{-1}$) H I absorption in the blue-violet region whereas the M spectrum dominates in the H α region. Both strong ultraviolet continuum and low-excitation absorption lines have been observed. They also observed flickering of up to 0.2 mag on a timescale of a few minutes and suggested that MWC560 is a symbiotic-like binary containing an M giant and a compact companion. The system loses matter by a highly variable high-speed wind from the compact star.

Previous observations have shown that the star's brightness is variable—in 1973 it was ~ 12.5 mag (ref. 2), and in 1984, ~ 11 mag (ref. 3).

We carried out our observations with the Coudé spectrograph of the 2-m telescope at the Bulgarian National Observatory Rozhen. The dispersion of the spectrograms, obtained on 103a0 emulsion is 18 Å mm^{-1} and the region covered was 3,600–4,900 Å. The spectra were digitized using a Joyce Loebel MDM6 microdensitometer and processing was performed on a PC-XT computer using the software ReWiA⁵. The UBv (ultraviolet–blue–visual) observations are performed with single-channel photometers attached to the 60-cm telescopes at the Rozhen and Belogradchik observatories.

Table 1 summarizes our estimates of the brightness of MWC560 in V band and for the colours B–V and U–B. The observations are reduced to a standard UBv system, but are not corrected for atmospheric extinction because of small angular distance between MWC560 and the standard star HD59380. The night-to-night changes in the V band are ~ 0.2 mag and the maximum difference over the set of observations is ~ 0.4 mag. Our monitoring of the ultraviolet with an integration time of 10 s confirms the presence of rapid variations with a wide range of timescales (~ 10 min to ~ 1 h) and a maximum amplitude ~ 0.3 mag. The standard deviation of the random noise is ± 0.02 mag. The time-series analysis do not show any periodicity. Figure 1 shows an example of the flickering of MWC560.

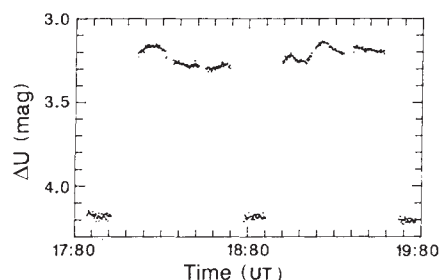


FIG. 1 The flickering of MWC560, observed on 5 March 1990 (top), compared to constant lower brightness of a standard star (bottom).

TABLE 2 Heliocentric radial velocities

Date Julian date (HJD24479...)	12 January 04.420	17 January* 09.468	3 February 26.380	6 March 57.307	12 March 63.270	14 March 65.313
Emission lines						
Metallic	+45 ± 1 (98)	+40 ± 1 (82)	+41 ± 1 (57)	+39 ± 1 (68)	+42 ± 1 (47)	
Hydrogen	+67 ± 3 (5)	+59 ± 4 (3)	+51 ± 4 (3)	+54 ± 1 (4)	+62 ± 4 (4)	
Absorption components of H I lines						
Fastest	-3,040 ± 50 (12)	-4,150 ± 60 (5)	-4,260 ± 70 (13)	-4,440 ± 30 (6)	-3,400 ± 25 (6)	-5,630 ± 10 (7)
Slowest		-290 ± 40 (3)	-210 ± 70 (3)	-270 ± 20 (4)	-270 ± 25 (4)	-240 ± 80 (5)
Sharp absorption				+51	+50	+49
Ca II K line				-44	-38	-50

The numbers in parentheses indicate the number of measured lines. The radial velocities are in units of km s^{-1} .

* Slightly defocused spectrum.

There are also significant changes in the continuum of MWC560. Recent observations^{6,7} show a large increase in the ultraviolet continuum, compared with 1984. The continuum in our spectra looks like that from a hot late B or early A star. We do not see any of the spectral features associated with an M4 giant. Other observations^{8,9,10} show that TiO bands are present in the red and infrared regions, but they are partially veiled^{8,9}.

The hydrogen lines of Balmer series dominate the line spectrum. Both emission and absorption components are observed but the former are measurable only for H β , H γ and H δ . They correspond to an almost constant radial velocity (Table 2) and moderate variations of the intensity. There are also many emission lines from, mainly, Fe I, Fe II, Cr II and Ti II. The corresponding radial velocity is also constant, but slightly lower than the velocity calculated from the hydrogen emissions. This is due to the influence of the low-velocity absorption components on the emissions in the profiles of Balmer lines.

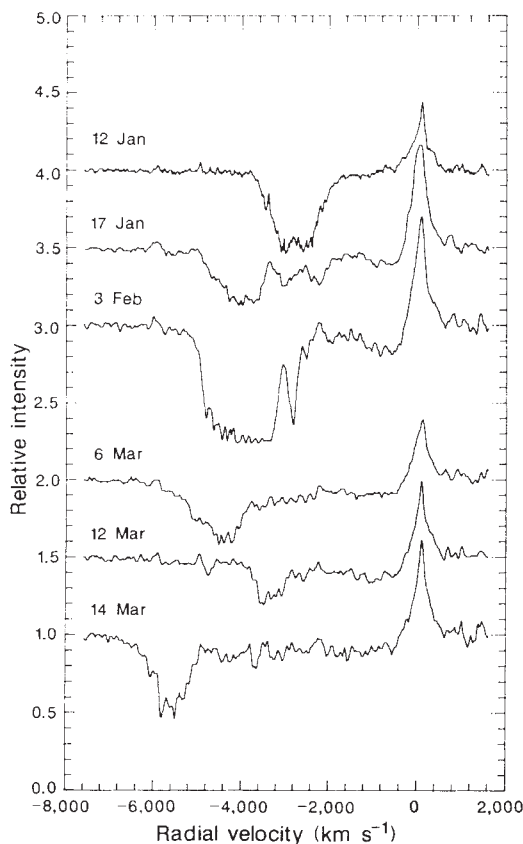


FIG. 2 Changes of the H β profile in the spectrum of MWC560 between January and March 1990. The spectra are normalized to the local continuum. The spectra for the different dates have been separated by adding a different constant shift to each one.

The system of absorption lines consists mainly of hydrogen lines and often shows multi-component structure (Fig. 2). The profiles of the components are very different from the usual stellar profile and represent the velocity distribution rather than the temperature and the pressure effects in the absorbing gas. When the lines have a simple shape they are visible up to H $_{20} \div H_{23}$. The equivalent width of the strongest H β component reaches 25 Å. Sometimes it is possible to measure the absorptions of the Ca II K line and He I 4,025 Å and 4,471 Å lines. They have the same radial velocity as the strongest hydrogen absorptions and an equivalent width of ~ 0.6 Å. Except for the hydrogen lines, only the sharp Ca II K line is present in absorption. It shows two components possibly of circumstellar and/or interstellar origin.

There is insufficient data to propose any detailed model of this object. Our data support the idea that MWC560 is a binary system comprising an M giant and a compact companion³ and having high rate of mass transfer. But our observations rule out the suggestion of the high-velocity stellar wind, due to absence of the specific wind-profiles. We propose that the matter transferred from the giant forms an accretion disk around the compact companion and that the emission lines originate in the outer parts of the disk. The lack of significant variation in the radial velocities calculated from the emission lines indicates that the disk is viewed face on. The strong changes in the Balmer absorption lines may be due to jet-like ejections along the line of sight. The absence of emission components with high radial velocities is evidence that these ejections are strongly collimated. The evolution of the hydrogen absorption lines may be due to dissipation on a timescale of a few days.

If the high-velocity Balmer absorption lines originate in the ejections we can compare MWC560 to another well-known jet-ejecting object, SS433. Both systems contain a main star and a compact companion. In the case of SS433 they are a massive OB star and a neutron star or black hole. In MWC560 the main star is a red giant. The observed velocities of these two objects differ by about a factor of 10 owing to the difference in ejection processes. We therefore speculate that the companion star in MWC560 is a less massive object—probably a white dwarf. Systems comprising an M giant and a white dwarf are quite common (as is well known from the evolutionary theory). The lack of any estimate of the distance to MWC560 and of data for the system geometry and the physical parameters of its components make it difficult to discuss this interesting object in more detail. □

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Io's atmosphere from microwave detection of SO₂

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SINCE the detection by Pioneer 10 of an ionosphere¹ around Io, this moon of Jupiter has been known to possess a tenuous atmosphere. The local observation of gaseous SO₂ and the upper limits on the concentrations of other gases deduced from Voyager data², together with the identification of SO₂ frost or adsorbate at the surface^{3,4} and upper limits on the global amount of atmospheric SO₂ (refs 5–8), have led to several classes of models for Io's atmosphere⁹. Here we report the microwave detection of SO₂ at 222 GHz, which constitutes the first direct global observation of Io's neutral atmosphere. The observations imply an SO₂ surface pressure of 4–35 nanobars, covering 3–15% of the surface on both leading and trailing sides of Io when illuminated by the Sun. This supports atmospheric models in which the partial pressure of SO₂ at the surface is determined by the local surface temperature⁸, favouring, in particular, the 'albedo cold-trap' models^{10,11}. Our failure to detect H₂S at 169 GHz suggests that the pressure of this gas is probably below 10^{−10} bar. Our results, taken together with the Pioneer ionospheric data, suggest that an atmospheric gas other than SO₂ is present. We propose that the locally buffered SO₂ atmosphere coexists with a background atmosphere of oxygen with a partial surface pressure of ~20 nanobars.

The observations were performed on 16–17 and 17–18 January 1990 with the 30-m IRAM (Institut de Radio-Astronomie Millimétrique) radiotelescope at Pico Veleta, Spain. The 42-h rotation period of Io allowed us to observe both the leading and trailing sides. We searched for the SO₂ line at 221.965 GHz and the H₂S line at 168.763 GHz using 100-kHz resolution. Because the on-line software corrected for geocentric but not joviocentric motion, we made numerous 2-min scans. Sky conditions were excellent (the zenith opacity at the SO₂ frequency was 0.1 on average), resulting in single-sideband system temperatures of ~400 K for SO₂ and 1,100 K for H₂S. Sky contributions were removed by 'wobbling' the secondary on and off the source at a frequency of 1 Hz.

Pointing was checked on Jupiter and, to test further the pointing accuracy and the calibration, scans on an 'anti-Io' sky position (with respect to Jupiter) were frequently recorded. Scans for which the continuum antenna temperature (the difference between the observed continua on Io and 'anti-Io' scans) was not consistent with the surface temperature of Io were not considered (~50% rejection).

Individual scans were corrected for Doppler shifts before co-addition. The accuracy of the velocity correction is estimated to $\pm 20 \text{ m s}^{-1}$, implying negligible broadening of the linewidth. Possible instrumental systematic effects that could have led to a smearing of the line were checked. The SO₂ line was detected with similar characteristics on both sides of Io. But a detailed examination of groups of scans corresponding to shorter (10–40 min) integration intervals revealed that the antenna temperature contrast of the line varied between ~0 K (non-detection) and 0.1 K (3 σ confidence level). The continuum check assured us that these variations were not caused by fluctuations in the pointing. When the line was detected, its linewidth (defined throughout as full width at half maximum (FWHM)) did not depart significantly from an average value of ~600 kHz. An exception to this occurred in the first six scans (12 min) of the second night (17 January 1990, 18.45–19.10 UT), which show

a much stronger (0.25 K) and narrower ($0.3 \pm 0.2 \text{ MHz}$) line than the average (Fig. 1). As a first step, we concentrated on the interpretation of the averaged line. Its width is $0.61 \pm 0.13 \text{ MHz}$ and its contrast, after correction for the antenna efficiency and for the filling factor of Io in the beam, is $18 \pm 7 \text{ K}$ at Io (Fig. 2). The H₂S data were reduced similarly and showed no line, with a 3 σ noise level of 50 K at Io.

The (11,11)–(10,0,10) transition of SO₂ at 221.965 GHz (lower energy level, 34.5 cm^{-1} ; absolute strength, $3.18 \times 10^{-22} \text{ cm molecule}^{-1}$ at 300 K; ref. 12) occurs in thermal absorption/emission conditions and is in local thermodynamic equilibrium at densities larger than 100 cm^{-3} (ref. 13). A typical upper limit on the pressure of $\sim 10^{-6} \text{ bar}$ has been inferred from stellar occultation¹⁴. As more stringent upper limits for a number of plausible condensable species have been set by Voyager at the hot spot where SO₂ was detected², this maximum value should hold at any location on Io; therefore, the SO₂ transition is purely Doppler broadened (Doppler FWHM, 220 kHz at 120 K; Lorentz FWHM $\leq 6 \text{ kHz}$).

As a first step, we modelled the line using a surface temperature of 130 K and emissivity of 0.9. The atmospheric contribution was specified by a single temperature T_{atm} and the fractional area of the disk from which the emission originates. In this model, the unconvolved observed linewidth (600 kHz) can be solved exactly for the line-centre opacity τ and SO₂ column density, which we express as a partial pressure p , assuming an airmass of 1 (which should hold for the conditions that,

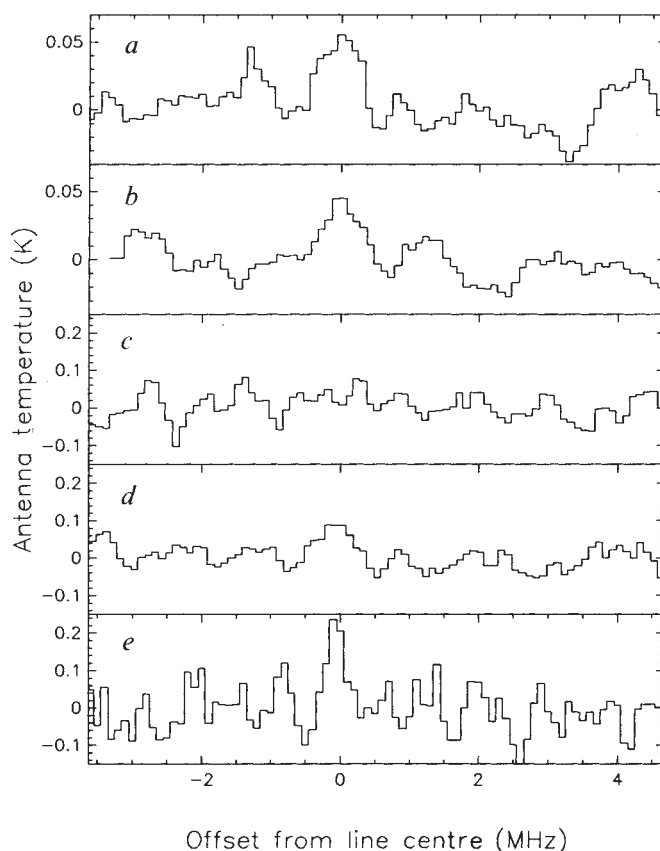


FIG. 1 Observed 221.965-GHz SO₂ line on Io. *a*, Leading side (17 January 1990, 0:00–03:00 UT). Integration time, T_{int} , of 79 min. *b*, Trailing side (17–18 January 1990, 18:45–02:45 UT) $T_{\text{int}}=129 \text{ min}$. *c*, Leading side (17 January 1990, 0:00–1:20 UT) $T_{\text{int}}=32 \text{ min}$. *d*, Leading side (17 January 1990, 1:31–2:45 UT) $T_{\text{int}}=35 \text{ min}$. *e*, Average of first six scans recorded on the trailing side (17 January 1990, 18.47–19.09 UT), $T_{\text{int}}=12 \text{ min}$. The only treatment on the scans is correction for Doppler velocity of Io around Jupiter, coaddition, and removal of the continuum (zero-degree baseline).

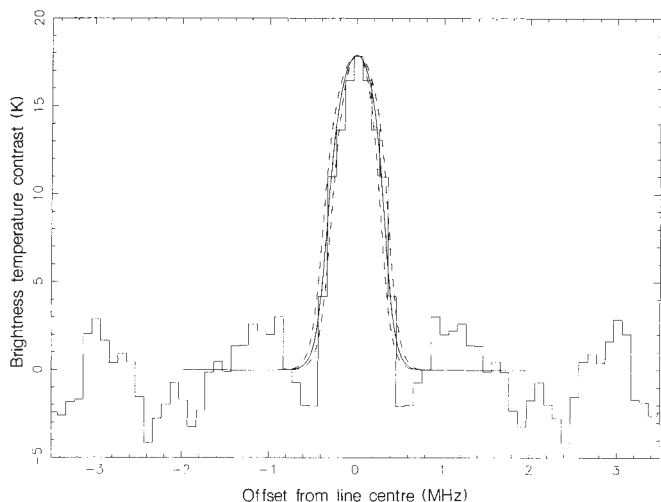


FIG. 2 Observed average SO_2 221.965 GHz line at Io, corresponding to 208-min integration. The data have been folded and corrected for the antenna efficiency (main-beam efficiency 0.45) and the filling factor in the ~ 13 -arcsec beam (1/175). The synthetic profiles were calculated with a Bates¹⁸ profile using a surface temperature of 130 K, an exospheric temperature of 700 K and an airmass of 1. Solid line: best fit, obtained for SO_2 pressure of 14 nbar. The linewidth is fitted, and the contrast is scaled to the observed value by a factor of 0.053. Dashed lines: SO_2 pressure of 6 (inner curve) and 35 (outer curve) nbar, rescaled by factors 0.069 and 0.044 respectively.

we believe, are most representative of Io). The optically thin case corresponds to $T_{\text{atm}} = 910$ K, $\tau = 0.02$ and $p = 1$ nbar. But this case, and all situations with $\tau \leq 1$, are implausible because they imply large values of T_{atm} (≥ 650 K) just above the surface. The extreme optically thick regime is reached for $T_{\text{atm}} = 135$ K and $p = 5.6$ nbar. In this case, the distribution of SO_2 gas over the disk is unconstrained. In a more typical intermediate case, $T_{\text{atm}} = 300$ K, the linewidth corresponds to $\tau = 6$ and $p = 4.1$ nbar. The line contrast produced in this situation, however, is 182 K; thus, only $\sim 10\%$ of the surface experiences such conditions. Pressures of 3–10 nbar are obtained for T_{atm} in the range 135–650 K, with the atmosphere covering 5–100% of Io's surface. If, as is likely, $T_{\text{atm}} \geq 220$ K, less than 20% of the surface is covered by such an atmosphere. Although this crude analysis will be modified quantitatively by the true shape of the temperature profile, it is clear that the SO_2 partial pressure is in the range of a few nanobars and that the atmosphere is probably confined to a small portion of the satellite. In contrast, the characteristic temperature of the atmosphere is not well constrained by the observations.

Both the ionospheric measurements¹ and current aeronomic models^{15,16} indicate that Io's thermal structure must be essentially thermospheric. Following Summers¹⁷, we used Bates¹⁸ description of the thermal profile, with the exospheric temperature T_{exo} as a free parameter. For several values of T_{exo} , the thermal profile was calculated and the observed lineshape analysed with a multilayer radiative-transfer code¹⁹, with the atmospheric filling rate (fraction of the surface covered by the atmosphere) of Io being adjusted to fit the line contrast. This, together with a test of Kumar's¹⁶ profile, shows that the SO_2 partial pressure is relatively insensitive to the exact temperature profile, the largest uncertainty coming instead from the experimental noise (Fig. 3). The SO_2 pressure is 12 nbar (number density $N = 7 \times 10^{11}$ molecule cm^{-3}), within a factor of three. If T_{exo} is larger than 400 K—scaling of the Pioneer 10 scale heights¹ by a mass of 33 (S^+ (ref. 15) or O_2^+) gives temperatures of 605 K and 195 K at 100 km for entry and exit—this atmosphere covers only 3–15% of Io's surface. Averaged over the surface, the SO_2

column density is 0.0015 cm amagat (4×10^{16} molecule cm^{-2}), within a factor of two, much lower than all available upper limits.

Noting the similarity of the line on both sides of Io, the simplest interpretation of our observations is that Io's atmosphere is in sublimation equilibrium with the surface, leading to a significant atmosphere only near the sub-solar point (or the warmest point, wherever it lies), as proposed in locally buffered models⁸. Our SO_2 pressure, however, corresponds to equilibrium at 121 ± 4 K (ref. 20), a value significantly lower than the probable temperatures near the sub-solar point (130–135 K). This supports 'regional (or albedo) cold-trapping' models^{10,11}, in which the brightest and coldest small-scale regions control the gas abundance, and which predict pressures of ~ 7 nbar at the sub-solar point¹⁰. It is difficult to assess the validity of the SO_2 flow models²¹, which predict departures from the locally buffered pressures by factors of only two. In contrast, 'sub-surface cold-trap' models²² (predicting pressures of 10^{-12} – 10^{-15} bar) are readily excluded by this observation.

A preliminary curve-of-growth analysis of the enhanced line seen in the early scans of the second night indicated that, for a short time, the SO_2 atmosphere was characterized by a lower SO_2 pressure and a much larger filling factor ($\sim 80\%$ of Io's disk). For these perturbed spectra, the total amount of SO_2 must have been about three times larger than under average conditions. This amounts to a surplus of $\sim 4 \times 10^{11}$ g of SO_2 (equivalent to a 1×10^{-6} -cm-thick layer over a hemisphere), which then must have dissipated on a timescale of ~ 10 min. As the free-fall time from a height of 20 km (~ 1 scale height) is only 2.5 min, we speculate that shortly after our observations started, a source located away from the sub-solar region cut off abruptly, leading to a rapid collapse of the SO_2 component of the atmosphere to the cold surface.

H_2S does not condense at temperatures > 90 K (ref. 23). Assuming a uniform horizontal H_2S distribution over Io's disk, the 50-K contrast limit implies a maximum H_2S ground pressure of $\sim 8 \times 10^{-11}$ bar (column density 1.7×10^{-4} cm amagat), unless the characteristic atmospheric temperature is less than ~ 170 K, in which case the (unobserved) H_2S line centre could be optically thick and less stringent upper limits could apply. This upper limit, much lower than previous ones², suggests that the absorption features recently identified^{24,25} at $3.9 \mu\text{m}$ are formed by H_2S embedded in the SO_2 ice and originated from a time-variable source²⁴.

We briefly examined Io's atmosphere thermal budget, adapting an aeronomic model recently developed for Titan²⁶. We considered only solar heating, which is probably the main energy input^{15,16}, and assumed a heating efficiency of 0.3. Unlike

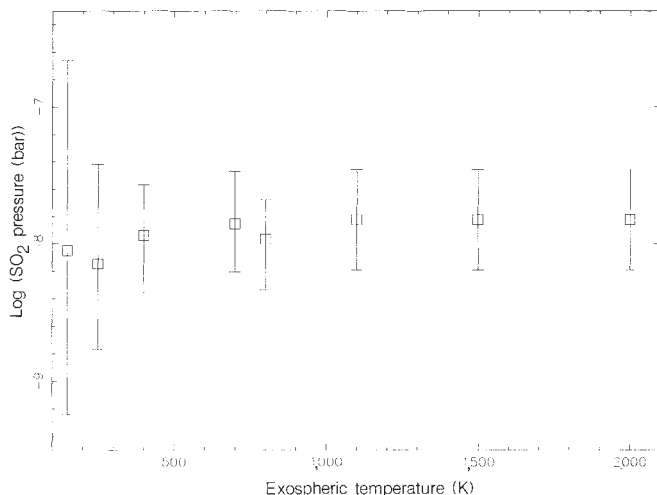


FIG. 3 Inferred SO_2 partial pressure, using mainly Bates¹⁸ profiles with several exospheric temperatures and an airmass of 1. For the 800-K point, Kumar's model¹⁶ was used.

Kumar^{15,16}, we used literature values¹³ for the relaxation rates of the ν_2 and (ν_1 , ν_3) systems of SO₂. These rates are much larger than that of CO₂, especially at low temperatures, and may, for high values of the SO₂ surface pressure, lead to a mesopause.

The SO₂ pressure at the terminator (the point at which the Sun is at the horizon) is unlikely to exceed the vapour pressure at 100 K, 4×10^{-12} bar. The corresponding exospheric temperature is <150 K. Thus, despite the complications associated with the Pioneer occultations⁹, the observed ionospheric scale heights probably imply another atmospheric constituent. H₂S has been proposed as a major constituent²⁷, but our upper limit rules it out as a stable background gas, although a transient H₂S atmosphere remains possible²⁴. Among other plausible candidates (O₂, CO, N₂, CH₄), O₂ has been advocated²⁸ as a non-condensable photolysis product of SO₂. In addition, O₂ is the most able to produce high exospheric temperatures because its dissociation threshold occurs at longer wavelengths (2,423 Å) and because it has no cooling far-infrared bands. Exospheric temperatures of 400 and 1,300 K are obtained for oxygen ground pressures of 5 and 40 nbar, respectively. We therefore propose that Io may have background atmosphere of ~20 nbar of O₂. Such a pressure would induce a 2% attenuation of the stellar flux under the conditions of the occultation reported by Smith and Smith¹⁴. Although this is below the detection threshold of their experiment, it could now be detected in occultation light curves of bright stars. Such an oxygen atmosphere could limit the transport of SO₂ away from sub-solar regions and inhibit the formation of polar caps²⁸. The net effect of regional cold trapping of SO₂ on the transport of frost to the poles is still unclear^{11,29}.

Although it is tempting to construct O₂-SO₂ aeronomic models of Io to simultaneously fit the ionospheric temperatures and the line presented here, the very high relaxation rate of the ν_2 band of SO₂ (pure¹³ and in O₂ (ref. 30)), which dominates the cooling at low temperatures, makes the cool-to-space approximation (direct radiation to space of infrared heat) incorrect in the first 40 km of the atmosphere. Detailed heat-transfer models, and a reassessment of ionospheric models, will be necessary to provide a more detailed picture of the atmosphere of Io. □

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Emission of charged particles from indentation fracture of rocks

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It has long been realized that earthquakes are often associated with various kinds of anomalous electromagnetic phenomena. These phenomena might be caused by rock failure in the Earth's crust, but the details of the mechanisms are still unknown. Here we describe an attempt to measure the emission of charged particles from rocks undergoing indentation fracture under atmospheric conditions, using a specially designed indentation system. High electron and ion emission intensities could be detected during parts of the loading cycle when cracking occurred around the indent. The emission behaviour of feldspar and quartz in granite was different from that of the hygroscopic matrix of hornblende andesite; moisture in the hornblende andesite enhanced emission appreciably. The different behaviour observed may be attributable to two different mechanisms.

Recently, the role of the emission of charged particles by rock fracture in the production of electromagnetic radiation associated with earthquakes has attracted much attention¹⁻³. It has been confirmed⁴⁻⁸ that the fracture of a wide variety of synthetic materials is accompanied by the emission of charged particles, but there have been few studies of fracture emission from rocks. Further, most of these studies have been made in vacuum because of the operational limitation of electron detectors.

A conventional hardness tester was equipped with an electroconductive Rockwell-type indenter, which served as an electrode to collect charged particles during indentation. The signal was detected using a high-sensitivity charge amplifier with a high-pass filter, allowing detection of spontaneous emission of charged particles as displacement current. The details of the measuring system are described elsewhere⁸.

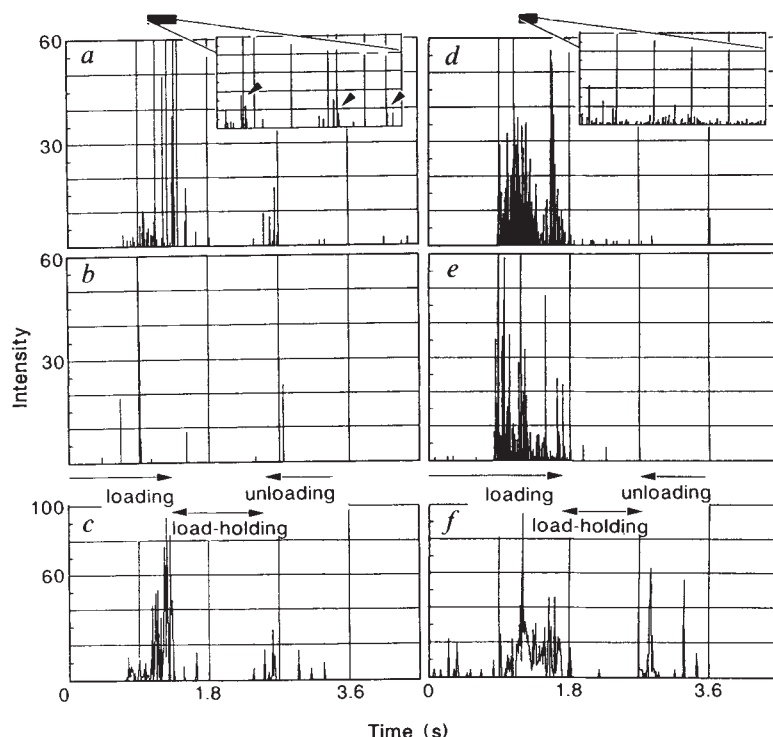
We tested eleven kinds of rock with applied loads ranging from 9.8 to 490 N and at temperatures up to 400 °C. Figure 1a, b shows typical emission signals from charged particles during fracture of feldspar. Figure 1c shows acoustic signals obtained during the same test as in Fig. 1a. During the loading half-cycle, frequent acoustic signals were recorded, and intense signals from electrons and negative ions were concentrated in the same period. When the signals during the loading half-cycle are displayed on an expanded scale (Fig. 1a inset), it is apparent that there are several bursts of fracture emission—in each, the peak intensity is followed by a decay on a timescale of ~10 ms (indicated by arrows). These bursts probably originated in the median-type transgranular cracking underneath the contact zone⁸.

Calibration tests using a standard charge generator indicated that the total amount of negative charge generated during the loading half-cycle was $\sim 2.3 \times 10^{-12}$ C, about 3.7 times the amount of positive charge generated, $\sim 6.2 \times 10^{-13}$ C.

During the period of maximum load, little fracture or acoustic (Fig. 1c) emission was detected, indicating that no cracking was occurring. During the unloading, some emission peaks again appeared, as the lateral cracking owing to the release of the residual stress occurred.

Figure 1d, e shows typical signals of both negatively and positively charged particles from a moisture-saturated matrix of hornblende andesite, and Fig. 1f shows acoustic signals obtained during the same test as in Fig. 1d. Signals from both negatively and positively charged particles appeared in rapid succession during loading. Unlike Fig. 1a, the inset in Fig. 1d

FIG. 1 Typical signals (arbitrary units) at room temperature of *a*, electrons and negative ions (the indenter bias, V_i is +15 V), *b*, positive ions ($V_i = -15$ V) from fracture of feldspar from granite during indentation at a load of 294 N and *c*, acoustic emission signal obtained in the same test as in *a*. *d*, *e*, Fracture emission and *f*, acoustic emission from matrix of hornblende andesite.



shows regular bursts with a frequency in the range 300–600 Hz. The integrated intensity of negatively charged particles over the loading half-cycle in Fig. 1*d* corresponds to a charge of $\sim 4.7 \times 10^{-11} \text{ C s}^{-1}$, 1.3 times larger than that of positively charged particles.

Because the behaviour of granite (anhydrous) and hornblende andesite (hygroscopic) differed, we made measurements of the

emission of negatively charged particles from rocks with different water content. Figure 2 shows the intensity integrated over the loading half-cycle plotted against water content. Among the dried rocks, the emission from granite was largest, and the emission from quartz was higher than that of feldspar. By contrast, less emission was noted from fractures of a dried hornblende andesite and quartz basalt. When the moisture

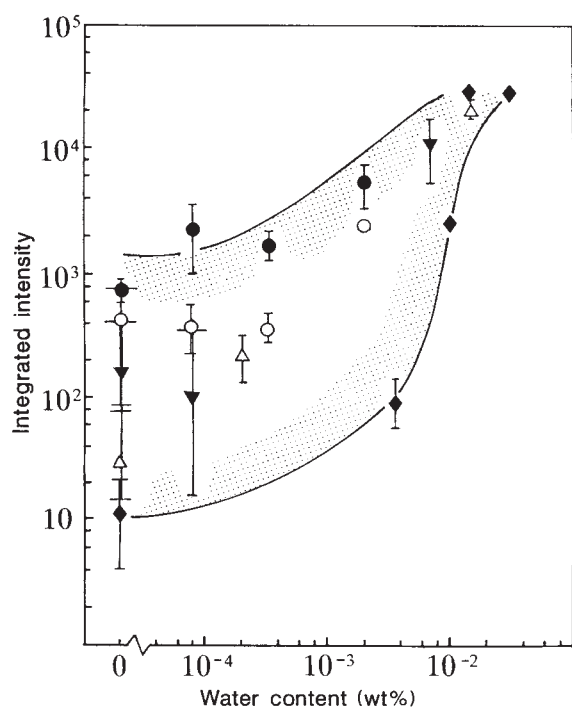


FIG. 2 Integrated emission intensity (arbitrary units) of electrons and negative ions during loading half-cycles as a function of moisture content of various rocks: ●, quartz from granite; ○, feldspar from granite; △, quartz basalt; ▼, alkali olivine dolerite; ◆, hornblende andesite. Indentation load, 294 N. Room temperature.

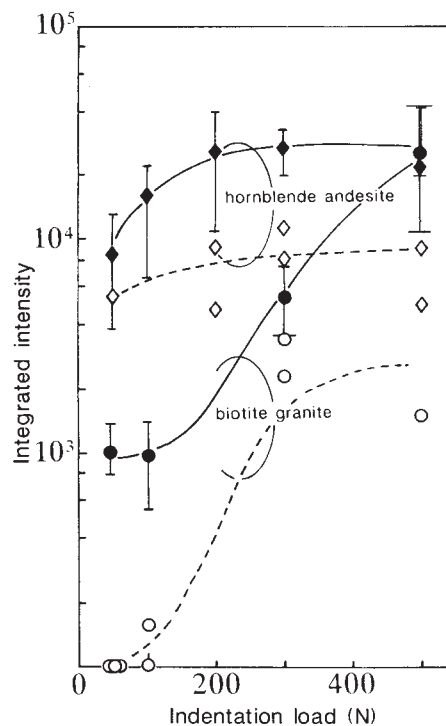
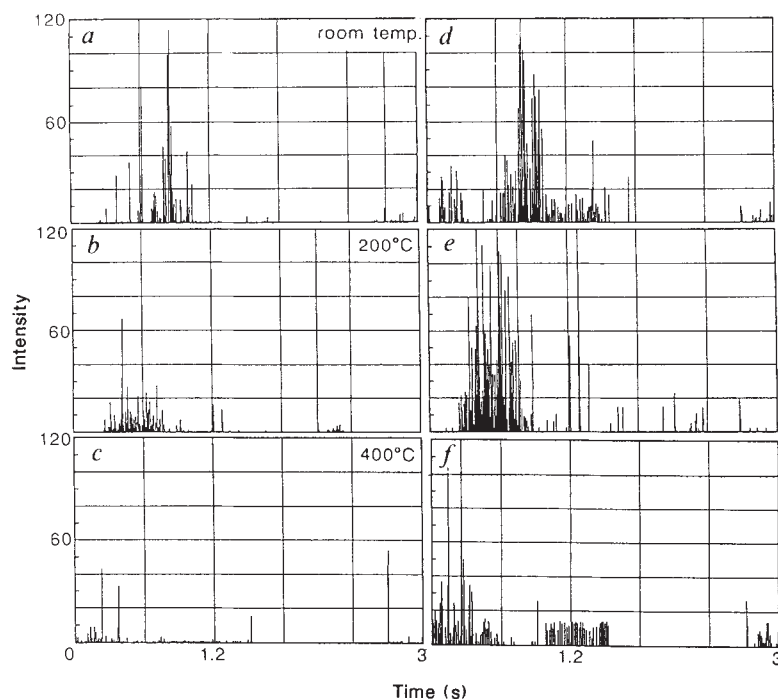


FIG. 3 Integrated intensity of electrons and negative ions (open symbols) and that of positive ions (filled symbols) from fracture of granite and water-containing hornblende andesite as a function of indentation load at room temperature.

FIG. 4 Emission of electrons and negative ions (a-c) and acoustic emission (d-f) signals from fracture of granite at elevated temperatures up to 400 °C. Load, 294 N.



content in andesite, dolerite and basalt was $\geq 10^{-2}$ wt%, however, emission from these rocks was as much as 100–1,000 times that of the dried rocks. At around 5×10^{-2} wt%, the intensity was comparable to that of rocks whose surfaces were entirely wetted. Because granite is anhydrous its fracture emission cannot be greatly increased.

We also studied the dependence on the applied load of the fracture emission from biotite granite and hornblende andesite (Fig. 4). The emission from andesite is higher because the specimens absorbed moisture and were easily fractured even at lower load. The integrated intensity of electrons or negative ions from the matrix of andesite was about twice as large as that of positive ions. There is less emission from the fracture of quartz in granite at lower loads, but for higher loads, 294–490 N, severe fracture occurred near the point of application and higher intensities were noted. Again, there was more emission of negative charge than of positive charge.

Typical signals of electrons and negative ions from granite at elevated temperatures of up to 400 °C are shown in Fig. 4, as well as corresponding acoustic signals. The emission was highest at room temperature and 200 °C; at 400 °C the intensity was only one-fifth of that at room temperature. The emission from andesite (not shown) was lower at temperatures > 50 °C, because of moisture loss. Thus elevating temperature does not increase the fracture emission of rocks over our experimental conditions.

Different emission mechanisms may be responsible for the difference between the fracture emission of anhydrous and hygroscopic minerals. One possibility is that charge separation on fractured surfaces produces high electric fields of the order of 10^6 – 10^7 V cm $^{-1}$, causing the field emission of electrons⁹. The energy of the emitted electrons may be in the keV range because of the acceleration in the strong electric field^{4,9,10}. The behaviour of anhydrous minerals such as quartz and feldspar can most likely be explained by this mechanism because cleavage cracking of a single crystal is effective in generating high charge densities on fracture surfaces and thus can produce high electric fields. The electron energy, however, was not measured in our experiments. High-energy electrons are also capable of ionizing nearby atoms and molecules by inelastic collisions.

The behaviour of hornblende andesite, alkali olivine dolerite and picrite basalt, however, are not attributable to the above

mechanism because these rocks show little emission unless they have a certain moisture content. The emission from these rocks may be due to the chemisorption of chemically active species such as water and oxygen on the charged layer of fractured surfaces. Excitation and dissociation of the molecule could then occur, producing free electrons and ions^{7,11}.

We now discuss the amount of charge generated by rock fracture, using hornblende andesite as an example. Indentation fracture of moist andesite produced a net negative charge of $\sim 1.2 \times 10^{-11}$ C s $^{-1}$ during the loading half-cycle at a load of 294 N. The volume of the fractured zone, estimated from the size of the indent, was $\sim 0.02 \times 10^{-9}$ m 3 . Thus the net production rate would be ~ 0.6 C m $^{-3}$ s $^{-1}$. If a massive failure occurs during about one second at ground level, over an area extending some metres, the charge generated may be comparable to the total electric charge produced by one bolt of lightning (~ 1 C). This may be sufficient to cause geoelectromagnetic disturbances and is particularly likely to be noticed at ground level under wet conditions. In the 1965 Matsushiro earthquake swarm, where many cracks and fissure zones were formed at the surface¹², luminescence was observed, as well as an anomalous change in Earth potential^{13,14}. Such surface cracking might cause emission of charged particles, which may be responsible for excitation of luminescence as suggested by Brady and Rowell⁴.

Further experiments to shed light on how fractures in the deep Earth can cause effects at the surface are now underway. □

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Analogues of epithermal gold-silver deposition in geothermal well scales

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ACTIVE geothermal fields are generally recognized¹⁻⁵ as modern analogues of the systems responsible for the formation of epithermal gold-silver ore deposits. We have investigated this analogy by studying scales precipitated at various depths in a geothermal well containing boiling fluids. Our results, presented here, show that concentrations and distributions of gold, silver, copper, lead and zinc in well M-53 at Cerro Prieto, Mexico, are comparable to those found in economic epithermal vein deposits, and that the highest concentrations of metals occur at or near the fluid production horizon. The onset of vapour separation or flashing at these depths in the well indicates that boiling caused sequential base and precious metal saturation through the loss of CO₂ and H₂S, respectively, to the vapour phase. These phenomena provide direct evidence that boiling of chloride-rich hydrothermal solutions may be responsible for the deposition and zonation of gold, silver, copper, lead and zinc in epithermal vein deposits. Geothermal well scales can thus provide the opportunity to study epithermal ore genesis in a wide variety of natural environments.

Although well scaling is a problem in the exploitation of geothermal energy, scales are potentially of great value in studies of the geochemistry of mineralizing processes. Scales from surface installations have been reported to contain significant Ag, Cu and Fe at Salton Sea, California⁶, to host a variety of iron and base metal sulphides at Cheleken, Soviet Union^{7,8}, and Milos, Greece⁹, and to contain locally abundant Au, Ag and Cu at Ohaaki and Kawerau, New Zealand¹⁰. High concentrations of gold, silver and base metals have recently been found in well scales collected from various depths in the Cerro Prieto¹¹⁻¹³, Oku-Aizu (Japan)¹⁴ and Salton Sea¹⁵ geothermal fields. The distributions as a function of depth and depositional mechanisms of these metals, however, have not been evaluated. The discoveries of metalliferous scales are of particular importance as they provide a means of investigating mineralizing processes under known or controlled conditions in natural hydrothermal systems. Such processes are generally inferred from studies of mineralization^{5,16,17}, constrained by laboratory experiments¹⁸, or simulated by computer models¹⁹⁻²³. The chemical zonations reported here can be directly related to those of fossil ore deposits and some of the physico-chemical controls of their formation.

Well M-53 at Cerro Prieto is 1,997-m deep and intersects the producing aquifer between 1,845 and 1,996 m. Between 1974 and 1978, the well operated intermittently under variable but generally high-pressure conditions in a testing mode. From 1978 the well steadily produced liquid and vapour under relatively low well-head pressure until closure for maintenance in 1982. During this production phase, scales accumulated on the well liners. These scales occur mainly between 920 m and the bottom of the well and, to a lesser extent, near the top of the well; the former precipitated during the production period when pressure conditions were low, and the latter during the high-pressure pre-production phase. Table 1 provides data on typical fluid compositions and well conditions during the production period. These data indicate that the fluid temperature in the producing aquifer was 337 ± 10 °C (calculated using the Na-K-Ca geothermometer), and the pH was 5.9 (estimated from CO₂ and HCO₃⁻ discharge data). Extrapolation of data for the H₂O-NaCl system²⁴ indicates that the fluid boiled in the well at a depth similar

TABLE 1 Typical liquid compositions and well-head conditions (1978-82)

Species	Concentration (mg kg ⁻¹)	Species	Concentration (mg kg ⁻¹)	Conditions
Na ⁺	9,563	Mg ²⁺	<1	<i>P</i> _{well-head} 7.4 bar
K ⁺	3,004	SiO ₂	1,351	pH _{wellbox} 7.2
Ca ²⁺	403	Cl ⁻	18,306	<i>X</i> _{vapour} 0.37
Li ⁺	27	HCO ₃ ⁻	79	Production rate
Rb ⁺	26	CO ₂ *	16,255	66 t h ⁻¹
B ⁺	24	H ₂ S*	770	

* Vapour composition.

to that of the production horizon. Assuming isenthalpic boiling, the drop in temperature was 140 °C.

Examination of polished thin sections indicates that scales at the top of the well consist of 0.1-0.5-mm-thick layers of clear to black botryoidal amorphous silica with ~10% of sulphide layers containing pyrrhotite and traces of sphalerite. The colour of the darker silica results from finely disseminated inclusions of a probable iron silicate phase²⁵. The proportion of dark silica gradually increases with depth. The percentage of sulphide decreases to <1% below 200 m and then increases sharply, composing >60% of samples below 1,100 m. These sulphides consist mainly of isocubanite with irregular layers of sphalerite and galena, and are locally accompanied by up to 0.5% silver-rich electrum (Fig. 1). Below 1,600 m, scales consist mainly of sulphide. Traces of calcite occur at irregular depths in the well.

As might be expected from the observed sulphide abundances, analyses of Cu, Pb and Zn show slightly elevated values near the top of the well which decrease with depth down to 890 m, and then sharply increase between 1,063 and 1,189 m (Fig. 2a). All three metals display similar distributions. The highest values occur near the bottom of the well (Cu, 12.87 wt%; Zn, 8.88 wt%; Pb, 8.28 wt%). Silver grades are high throughout the well (>280 g tonne⁻¹), but increase abruptly below 890 m to reach a peak of 22,900 g tonne⁻¹ at 1,349 m; silver contents are markedly lower towards the bottom of the well (Fig. 2b). The profile of gold values is similar. Gold concentrations are low at the top of the well (0.11 g tonne⁻¹) and rise gradually to a depth of 1,063 m (Fig. 2c). Below 1,063 m, gold increases sharply to a maximum value of 270 g tonne⁻¹ at 1,487 m, and then decreases rapidly further down the well.

The most striking feature of the metal distributions in Fig. 2 is that the gold and silver concentrations peak 365 m and 495 m above the top of the production horizon, respectively, whereas the Cu, Pb and Zn concentrations are highest near the bottom of the well. In solutions with physico-chemical characteristics similar to those of well M-53 fluids, gold is transported dominantly as a bisulphide complex^{26,27}, and silver mainly as a chloride complex above 300 °C and as a bisulphide complex

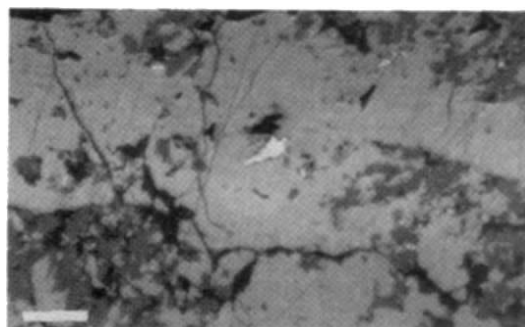


FIG. 1 Photomicrograph of silver-rich electrum (white), isocubanite (light grey), sphalerite (dark grey) and amorphous silica (black) in scales from 1,349-m depth in well M-53, Cerro Prieto. Scale bar, 25 µm.

at lower temperatures^{28,29}. Copper is transported as a chloride complex at aquifer conditions, but bisulphide complexing may be important at lower temperatures³⁰. Lead and zinc solubilities are dominated by chloride complexing for all relevant temperatures^{31,32}.

As noted above, boiling in the well starts at a depth similar to that of the production horizon. The principal effects of such phase separation on the liquid are to decrease temperature, increase pH and decrease H₂S activity. Most of the increase in pH occurs within the first few per cent of boiling, whereas significant loss of H₂S requires a more advanced degree of boiling²⁰. Chloride complexes are destabilized by decreasing temperature and hydrogen ion activity. By contrast, the stability of bisulphide complexes is enhanced by the increased pH of the liquid (gold solubility increases by ~1 log unit per pH unit for the well conditions^{26,27}), and is relatively insensitive to temperature. Precipitation of sulphide minerals and resultant decrease of reduced sulphur species in the fluid may also affect bisulphide stability. Thus the principal cause of bisulphide complex instability is the loss of H₂S and resulting decrease in ligand concentration (HS⁻).

The distribution of gold and silver in the well scales demonstrates that these metals generally became saturated several hundred metres above the production horizon. For gold, this can be explained by the fact that significant depletion of H₂S in the liquid required considerable boiling. Silver deposition,

on the other hand, may have been controlled by the destabilization of both chloride and bisulphide complexes. If this were the case, precipitation of silver would have occurred in response to decreasing temperature and increasing pH, and to loss of H₂S respectively. But the remarkable similarity in the silver and gold concentration profiles (Fig. 2b, c) indicates that the transport of silver, like gold, was dominated by HS⁻ complexing in the well. Precipitation of the precious metals is thus primarily dependent on the partitioning of H₂S to the vapour phase during boiling.

Deposition of base metals is strongly controlled by temperature and pH. The estimated large drop in temperature and the decrease in hydrogen ion activity owing to boiling reduced the solubilities of Cu, Pb and Zn by several orders of magnitude. We therefore conclude that the Cu, Pb and Zn concentrations in scales near the bottom of the well were caused by boiling when high-pressure aquifer fluids entered the lower-pressure well environment. Localized base-metal-bearing scales near the top of the well are as yet poorly understood. These may reflect boiling near the top of the well in response to the high well-head pressures that prevailed during the pre-production phase.

The extreme concentrations and distinctive zonations of gold, silver, copper, lead and zinc in the geothermal well scales mimic those commonly described^{33,34} for epithermal Au-Ag and polymetallic vein deposits. Mineralization in many of these deposits is hosted by quartz veins precipitated from boiling hydrothermal fluids. Well M-53 seems to model an open-vein system in which ascending silica-saturated fluids boil and sequentially deposit base and precious metals. Although this analogy and type of zonation has been reported for mineralized geothermal fields¹⁷, it has not been previously recognized in well scales. As geothermal fields around the world tap compositionally diverse hydrothermal fluids under widely variable physico-chemical conditions, well scales should provide the opportunity to investigate the genesis of a broad spectrum of ore deposits. □

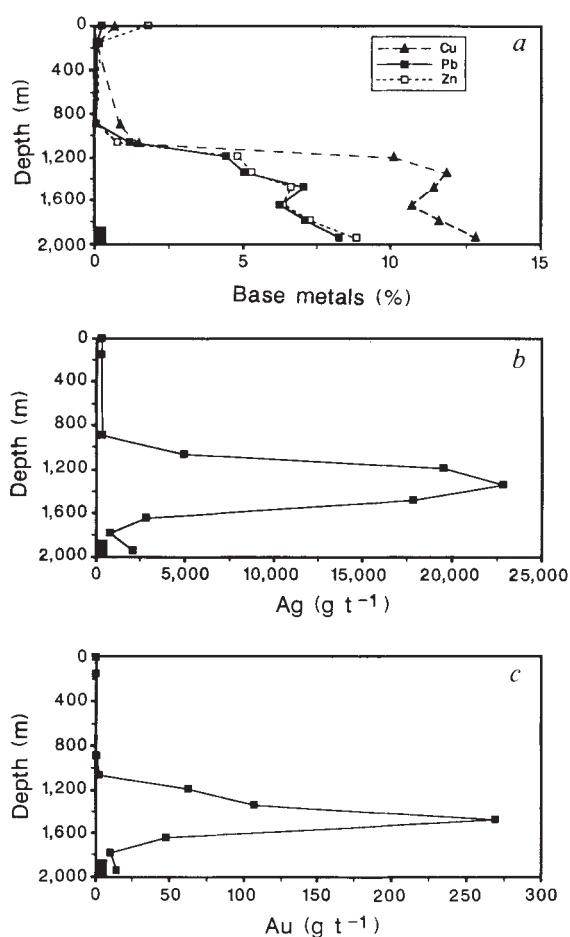


FIG. 2 Concentration profiles for copper, lead and zinc (a), silver (b) and gold (c) in well M-53, Cerro Prieto. Black bars between 1,845 and 1,996 m on the depth ordinates indicate the fluid production horizon, and level of initial boiling. Samples were analysed by inductively coupled plasma spectroscopy for Cu, Pb, Zn and Ag. Gold was analysed by a combination of fire assay and graphite-furnace atomic absorption techniques.

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Seismic images of a magma chamber beneath the Lau Basin back-arc spreading centre

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WE present normal-incidence seismic reflection profiles from a back-arc spreading centre in the southern Lau Basin, which show a bright, mid-crustal reflector beneath the ridge axis. Analysis of wide-angle seismic data collected simultaneously with the reflection profiles shows that the reflector coincides with the top of a low-seismic-velocity zone. We interpret the reflector as the top surface of a magma chamber. The Lau Basin is one of only two places where a magma body has been clearly imaged beneath a spreading centre, the other being the East Pacific Rise between 9° and 13° N (ref. 1). The Lau Basin reflector is imaged beneath an entire 35-km-long ridge segment. It is also present beneath both ridges and the overlap basin of a small overlapping spreading centre. This is the first observation of a widespread magma body beneath an overlapping spreading centre, and indicates that the offset here is not related to a break in continuity of the magma chamber.

The Lau Basin is a small back-arc basin associated with subduction at the Tonga Trench (Fig. 1a). In the south of the basin, two reconnaissance seismic reflection profiles collected by the US Geological Survey revealed a magma chamber reflector beneath the Valu Fa Ridge, the active sea-floor spreading centre². The ridge is located approximately parallel to and only about 20 km west of the eastern edge of the active Tofua volcanic arc. Lavas erupted at the ridge have an island-arc component and range in composition from andesitic basalt to andesite³. In July 1988, aboard RRS *Charles Darwin*, we collected 1,300 km

of normal incidence and wide-angle seismic data over the Valu Fa Ridge between 22°30' S and 22°10' S. Sea Beam bathymetry over this part of the Valu Fa Ridge⁴ shows that it consists of three en echelon segments—the southern, central and northern Valu Fa Ridges (SVFR, CVFR and NVFR). Each segment is 30–40 km long and they are separated by small (<5 km) non-transform offsets. Our survey area covers the whole of the central ridge and its overlap with the northern ridge. The data consist of 38 digitally recorded, four-channel reflection profiles crossing the axis at ~1-km spacing, and five profiles parallel to the axis (Fig. 1b). The seismic source was an array of four airguns totalling 22.8 litres. Most of the survey was acoustically navigated. Disposable sonobuoys were deployed on each of the five lines parallel to the ridge, and these recorded wide-angle arrivals at ranges up to 15 km, providing good control on the seismic velocity structure of the upper 3–4 km of the crust.

The seismic sections in Fig. 2 show two typical reflection profiles across the central ridge. In both sections, a bright, flat event between 4.2- and 4.3-s two-way time (TWT) can be clearly seen beneath the ridge crest. We believe this to be a reflection from the top of the crustal magma chamber. Amplitude analysis of the wide-angle data from the profile along the axis of the central ridge shows the presence of a crustal low-velocity zone, the top of which coincides with the magma chamber reflector (MCR) at a depth of 3 km beneath the sea floor. At the East Pacific Rise, the magma chamber reflection is shallower—between 1.2 and 2.4 km beneath the sea floor¹. The difference is probably attributable to the lower spreading rate⁵ of the Valu Fa Ridge (70 mm yr⁻¹ full rate, compared with 110 cm yr⁻¹ at the EPR) and the greater viscosity of the more intermediate magmas⁶. It implies significant differences in the crustal structure of the ridges themselves and the crust created at them.

The MCR varies in width between 1 and 2.5 km. We deduced this by carefully migrating the cross-ridge profiles, using a 45° finite-difference algorithm and a two-dimensional velocity field based on the results of the wide-angle profiles. The widths were

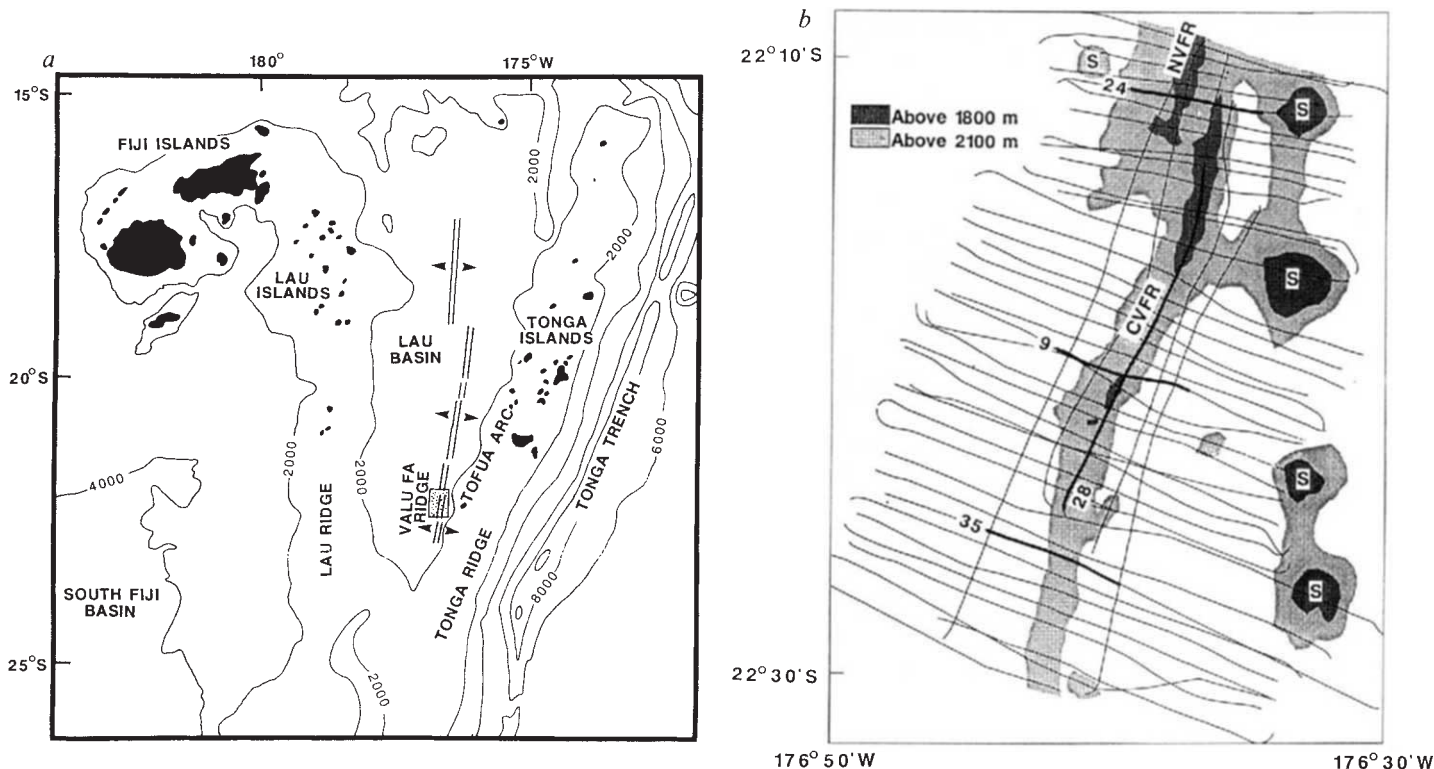


FIG. 1 a, Tectonic setting of the Lau Basin. The approximate positions of the spreading axis within the basin (shown by parallel lines and divergent arrows) are taken from refs 4 and 15. The location of the CD34/88 survey area is shown by the stippled box. b, Bathymetry of Valu Fa Ridge and

locations of seismic profiles (thin lines). Bold lines show sections of reflection profiles discussed here. The US Geological Survey profiles² cross the ridge axis at 22°18' S and 22°27' S. NVFR, northern Valu Fa Ridge; CVFR, central Valu Fa Ridge; S, off-axis seamounts.

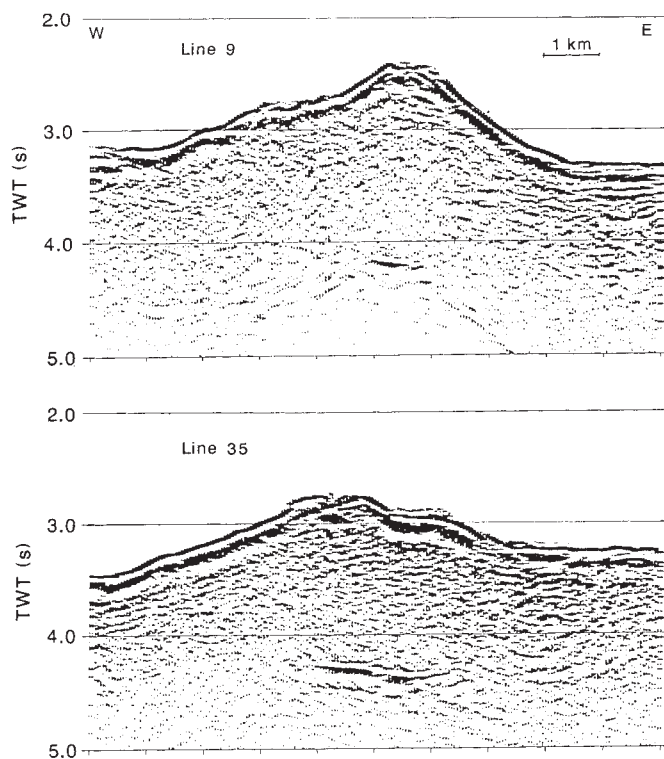


FIG. 2 Typical seismic reflection profiles across the CVFR. The traces have been stacked (fourfold), deconvolved, band-pass filtered from 4 to 20 Hz, 45° finite-difference migrated and plotted as a true-amplitude section. The bright crustal reflector between 4.2 and 4.3 s TWT beneath the ridge axis on both profiles is interpreted as the top surface of the magma chamber. Vertical exaggeration is 2.7 times at the sea bed.

then confirmed by forward modelling using two-dimensional ray tracing. By matching the prominent diffraction tails in the unmigrated data we were able to locate each edge of the reflector to within 200 m laterally. The presence of diffraction tails is indicative of the magma body having sharply defined, rather than gradational edges. Our analysis of the wide-angle data from the profile located 4 km west of the axis, on crust 114 kyr old, shows no evidence for a low-velocity zone in the top 4 km of the crust, confirming that melt is restricted to a narrow region. These results add to the growing seismic evidence^{7–10} for magma chambers that are significantly narrower than those predicted by studies of ophiolites^{11,12}. This dispels doubts that the results from the East Pacific Rise may not be typical of intermediate and fast spreading ridges. Further, the Valu Fa Ridge is in a

back-arc setting, where many ophiolites are believed to have formed.

We can calculate the reflection coefficient of the MCR by comparing the relative amplitudes of the sea-floor reflection, its first sea-surface multiple and the MCR itself and applying appropriate transmission-loss corrections¹³. This procedure gives a minimum reflection coefficient of 0.3–0.4, a very high value and comparable to that of the sea floor. Assuming a velocity of 5.3 km s⁻¹ based on the sonobuoy data for the material immediately above the interface, the observed reflection coefficients indicate P-wave velocities in the melt of 2.3–2.9 km s⁻¹. These velocities are at the lower end of the range of experimental measurements for basaltic andesite melts⁶. It is possible that the brightness of the event is due to constructive interference between reflections from the top and bottom of a thin sill-like layer containing a high proportion of melt, underlain by more-crystallized material. Such a model has been previously suggested on thermal grounds^{5,14} and proposed from seismic studies at the East Pacific Rise^{9,10}. A layer ~50–100 m thick would be consistent with the frequency content of this reflector, which is dominantly 10–12 Hz.

Figure 3 shows part of the reflection profile along the axis of the central ridge (line 28). The MCR is visible at TWTs of 4.1–4.3 s along the entire profile. It is also imaged on every one of the 38 cross-ridge lines. Despite this continuity, its brightness and character vary considerably. There is also a positive correlation on the cross-axis lines between the width of the MCR and the width of the overlying ridge. The results of our ray tracing confirm that both this correlation and the variations in MCR brightness are real, and not artefacts of the seismic imaging introduced by sea-floor topography. On the basis of these observations, we can recognize three distinct subsections of the central ridge. The southernmost third, exemplified by line 35, has a wide bright MCR and a broad ridge crest. The central section, crossed by line 9 and by line 28 between common depth points (CDPs) 120 and 320, has a narrow but bright MCR and a narrow ridge crest. The northern third between CDPs 340 and 520 on line 28 has a shallow and narrow ridge crest, underlain by a narrow low-amplitude MCR. We conclude that these three subsections, each 10–15 km long, are at different stages of a magmatic cycle. In the south the chamber is recharged before extrusive volcanism. In the centre the chamber has been partially discharged to form the narrow volcanic cone (above 2.7 s TWT on line 9). In the north, the chamber has been discharged almost completely during construction of the ridge summit, which is shallower here than anywhere else.

Nine of our lines cross the small overlapping spreading centre (OSC) system which separates the northern and central Valu Fa Ridges. One of these (line 24) is shown in Fig. 4a. The data are presented unmigrated, because the extreme horizontal velocity gradients, which are due to the shape of the sea floor in the

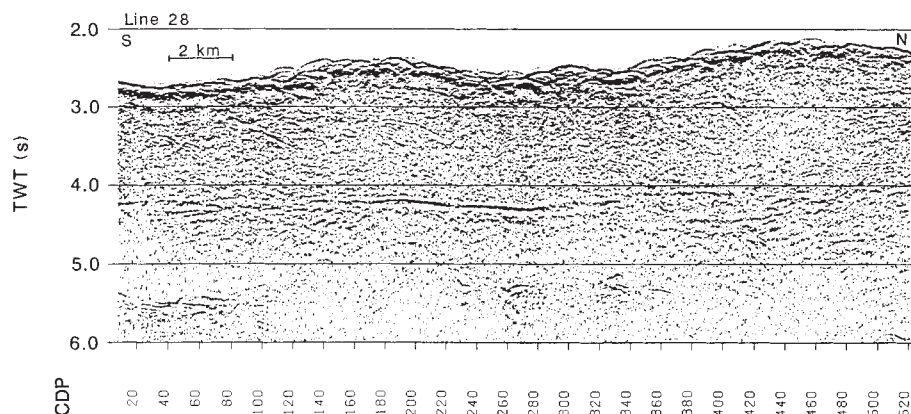


FIG. 3 Seismic reflection profile along the crest of the CVFR. The magma-chamber reflection is the continuous but variable amplitude event at 4.1 to 4.3 s TWT. The water-bottom multiple is a much weaker and more discontinuous event at a TWT of 5.1–5.6 s. Processing as Fig. 2 but without migration. Vertical exaggeration is 3.3 times at the sea bed.

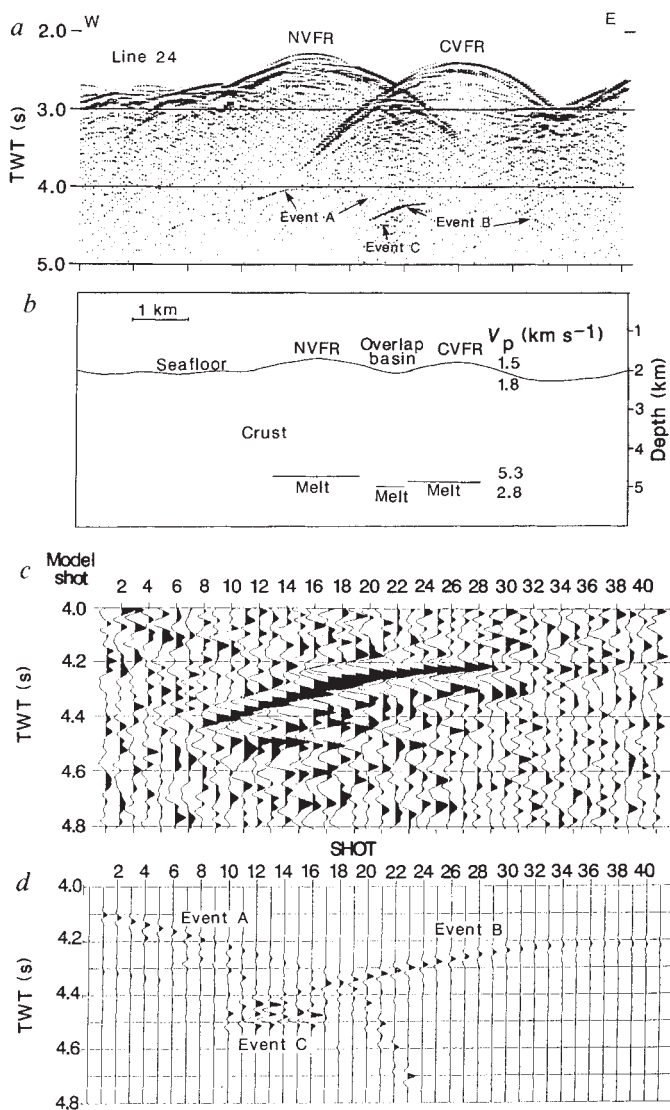


FIG. 4 *a*, Seismic reflection profile across the overlapping spreading centre. Processing as for Fig. 2, but without migration. *b*, Best-fit model derived from ray tracing of the distribution of magma-chamber reflectors. The model has a two dimensional velocity field, with isovelocity contours parallel to the sea floor. *c*, Detail of seismic reflection data beneath the overlap basin. *d*, Synthetic seismograms obtained by ray tracing through the model shown in *b*.

overlap basin, make migration unreliable. In Fig. 4*c* we show an enlargement of the seismic data from beneath the overlap basin. This region is characterized by diffractions (events A and B) that are due to the presence of melt beneath each of the two ridges, and a third event labelled C. We have used forward modelling to investigate how the melt is distributed. Figure 4*b*, *d* shows the best-fitting model that we were able to obtain using flat-topped magma bodies, and the resulting synthetic seismograms. Event C can be modelled only by the presence of a melt body beneath the overlap basin. We obtain similar results from modelling adjacent lines. We conclude that in the overlap region, melt is present beneath both spreading ridges, and also at least in some places beneath the overlap basin. The melt beneath the basin is consistently deeper by 100–200 m than that beneath the ridges. Because of the combination of sea-floor topography and the depth of the reflectors, we cannot resolve whether the melt body beneath the basin is continuous with those beneath the ridges. Given the close proximity of the three bodies, however, we think it likely that they are connected, and that a single magma body feeds both limbs of the OSC. □

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Cerebellar GABA_A receptor selective for a behavioural alcohol antagonist

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BENZODIAZEPINES are widely prescribed anxiolytics and anti-convulsants which bind with high affinity to sites on the GABA_A receptor/Cl⁻ channel complex and potentiate the effect of the neurotransmitter GABA (γ-aminobutyric acid)^{1,2}. The heterogeneity of benzodiazepine recognition sites in the central nervous system^{1,3,4} was revealed by studies showing different classes of GABA_A receptor subunits (classes α, β and γ)^{5–7} and variant subunits in these classes, particularly in the α-class^{8–11}. Expression of recombinant subunits produces functional receptors; when certain α-variants are coexpressed with β- and γ-subunits the resulting receptors have pharmacological properties characteristic of GABA_A-benzodiazepine type I or type II receptors^{6,11,12}. The α-variants are differentially expressed in the central nervous system¹³ and can be photoaffinity-labelled with benzodiazepines^{14,15}. Here we report a novel α-subunit (α6) of cerebellar granule cells. We show that recombinant receptors composed of α6, β2 and γ2 subunits bind with high affinity to the GABA agonist [³H]muscimol and the benzodiazepine [³H]Ro15-4513 (refs 14, 16) but not the other benzodiazepines or β-carbolines. The same distinctive pharmacology is observed with GABA_A receptors from rat cerebellum immunoprecipitated by an antiserum specific for the α6 subunit. We conclude that this α-subunit is part of a cerebellar receptor subtype, selective for Ro15-4513 (ref. 17), an antagonist of alcohol-induced motor incoordination and ataxia¹⁸.

The primary structure of the novel GABA_A receptor α-subunit (Fig. 1), designated α6, was predicted from cloned complementary DNAs isolated from bovine and rat cerebellar cDNA libraries on the basis of sequence similarity to other α-subunit cDNAs⁸. The sequence of the mature 434-residue polypeptide has 60% identity with the other α-variants and only 30% with β-, γ- and δ-subunits^{5–7}. The predicted extracellular domain specifies three N-linked glycosylation sites. The proposed

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intracellular region flanked by transmembrane segments 3 and 4 contains three consensus phosphorylation sites, two for protein kinase C and one for protein kinase A. These sites may be used by neurons to regulate GABA-gated chloride channel activity¹⁹. Interestingly, as determined by northern blot analysis (not

shown) and by *in situ* hybridization (Fig. 2), the expression of the $\alpha 6$ subunit in rat brain seems to be confined to the cerebellum. Within this part of the brain, the $\alpha 6$ messenger RNA (~2.7 kilobases) is selectively synthesized by the granule cells.

The pharmacological signature of the $\alpha 6$ subunit was determined using cultured mammalian cells engineered for the simultaneous transient expression of GABA_A receptor $\alpha 6$, $\beta 2$ and $\gamma 2$ subunits^{6,11,12}. Receptors were formed that had a benzodiazepine recognition site with vastly different pharmacological properties from the GABA_A-benzodiazepine type I-like site of homologous receptors that contain an $\alpha 1$ subunit¹² in place of $\alpha 6$ (Table 1). Specifically, [³H]Ro15-4513, a partial inverse agonist, was the only benzodiazepine that bound with high affinity to the receptors containing the $\alpha 6$ subunit. This binding was insensitive to competition by clonazepam, diazepam, flunitrazepam, 2-oxoquazepam, CL 218-872 and triazolam, but was displaced by Ro15-1788—the parent compound of Ro15-4513—at an inhibition constant K_i of more than 100 times those

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-60  GATCTGGCTGAGGGAGGAAGAAAGCAGGAGGCTGACCTGGCATTTCAGTGAACCATAGG
1    ATGCTGTTGCTTCTCCCTGGCTCTTCAGTTTACTATGGATAGAAATGCCAAGCTCAA
-19  M L L L L L P W L F S L L W I E N A Q A Q
    V S P C Y I V V L G K
61  CTTGAAGATGAAGGAACTTCTATTCTGAATGTGACGTCGGATTTTGACAACCTGCTG
2    L E D E G N F Y S E K N I V S R I L D N L L
121 GAGGGCTATGACACCGCTCTACGGCGGGATTTGGAGGTGCTGTAAACAGAGTTCAAAACA
22  E G Y D N R L R P G F G G A V E V K T
181 GATATCTATGTACACGCTTTGGGCTGTGTACAGATGTGGAGATGGATACAAATGGAT
42  D I Y V T S F G P V S D V E M E Y T M D
241 GTTTCCTCCGACAGACATGAGCTGATGAGAGCTGAAGTTCAAAGCGCTGCTGAGATT
62  V F F R Q T W T D E R L K F K G P A E I
301 TTGAGTTTAAATAACTTGATGGTCAAGATCTGGACTCCGGACACATTTTCCGAAAT
82  L S L N N L M V S K I W T P D T F F R N
361 GGGAAAAGTCAATTGCTCACAACATGACACCCCTAACAACTCTTCCGATTGATGCAT
102 G K K S I A H N M T T P N K L F R L M H
421 AATGGACGATCTGTACCATGAGGCTTACCCTCAAGCTGACTGTCGATGAGACTG
122 N G T I L Y T M R L T I N A D C -P-M--R-L-
481 GTTAACCTCCCTATGGATGGACACGCTGTCACCTCAAGTTTGGGAGCTATGCTATCCG
142 V-N--F--P--M--D--G--H--A--C P L K F G S Y A Y P
541 AAAAGCGAATCATATATACATGAAAAAGGACCGCTTTTTCAGTAGAGGTCCAGAA
162 K S E I I Y T W K K G P L Y S V E V P E
601 GAATCTTCAAGCTCCCTCCAGTATGATTGATTGGGCAACAGTTTCTAGTGAGACTATT
182 E S S S L L Q Y D L I G Q T V S S E T I
661 AATCGAACACAGTGAATATGTAATGACAGCTTCACTTCCACTTCAAGGAAGATG
202 K S N T G E Y V I M T V Y F H L Q R K M
721 GGCTATTTTCATGATCCAGATTTCACCTCCGTCATGACAGTCACTCTCTCAAGTG
222 G Y F M I Q I Y T P C I M T V I L S Q V
781 TCTTCTGGATTAAAGAGTCCGCTCCAGCAGAACCGCTTTGGAATCACCACGGTT
242 S F W I N K E S V P A R T V F G I T T V
841 TTAACCATGACCACTTAAGCATCAGTCTCGGCACTCTCTACCCAAAGTGTCTTATGCA
262 L T M T T L S I S A R H S L P K V S Y A
901 ACPGCCATGGATGGTTTCATAGCTGATGCTTTGGCTTTGCTTTCTGCTCTCATGAA
282 T A M D W F I A V C F A F V F S A L I E
961 TTCCGAGCTGTCACTACTTCAACATCTCCAGTCCGAGAAAGCGAAGGACGACAG
302 F A A V N Y F T N L Q S Q K A E R Q A
1021 ACTCGAGCCAAAGCCCGGTAGCAAAAGTCAAAACAACTGAATCACTGGAAGCTGAGATT
322 T A A K P P V A K S K T T E S L E A E I
1081 GTTGCACTCTGACTCCAAGTACCATCTGAAGAAGAGAACTGCTCTGCACTTTGCCA
342 V V H S D S K Y H L K K R I S S L T L P
1141 ATCGTTCCATCTTCTGAGCCGCAAGTCTCTGAGTGAAGCCCATCTTACCATCAACG
362 I V P S S E A S K V L S R T P I L P S T
1201 CCGGTCACTCCCCATTGCTTACCAGCCATTGGCGGACACAGCAAAATAGATCAATAT
382 P V T P P L L L P A I G G T S K I D Q Y
1261 TCTCGAATCTCTCCAGTAGCATTTCAGGAGTCAACCTTTGTGATGGATAGTTTAC
402 S R I L F P V A F A G F N L V Y W I V Y
1321 CTTTCCAAAGATACATGGAAGTGAAGTACTGTGAGTAGTTTCCAGCCAGGAAAAGC
422 L S K Q T M E Y S S T V E
1381 TGGAACACTGAATTTGGTTATTTTCCATTTTCTCGCTTACAGAACAAATATTGTGTT
1441 AGTATAAATGGTCTCATTTTCAGAACTAAATGTCTATATTTCATAATATGGAATAAGCA
1501 TGTTGGGCTAAAAACAATAATATTACTCTTCAAAATTAATTTAGAAAGCTACTGAAA
1561 TATGAATTTTCTGATAGTAAATTAATAATAATTAACAATAATATCAGCATTTATGT
1621 GGTAAGGTAGATTCAAAATGAGTTTGCCAACTTTGCTTCAATTTGCAATATTTTAA
1681 TTGTCACTGTGAATAACATTTTCCAAAGCAGATATATTGATATGTCGACCTTCTA
1741 AGCTT 1745

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FIG. 1 Nucleotide and predicted amino-acid sequence of the rat GABA_A receptor $\alpha 6$ subunit and amino-acid substitutions in the bovine homologue. The amino-acid sequence is shown (single-letter code) below the nucleotide sequence. Numbers on the left denote nucleotides and amino-acid residues. Negative numbers specify residues in the signal sequence and, for nucleotides, in the 5' untranslated sequence. Arrow, proposed end of the signal sequence²⁹. Putative N-linked glycosylation sites are boxed, the invariant extracellular cysteine-flanked loop structure is hyphenated, the four transmembrane regions are stippled and numbered on the side, and the putative protein kinase sites are circled. Open circles, consensus sites for protein kinase C; stippled circle, a site for protein kinase A. Bovine $\alpha 6$ amino-acid substitutions are indicated below the rat sequence. The C-terminal peptide sequence SKDTMEVNSVE used to generate the $\alpha 6$ antisera is underlined. METHODS. A rat cerebellar and bovine brain λ gt10 cDNA library were screened for GABA_A receptor α -subunits, using as a probe a DNA fragment derived from the bovine $\alpha 1$ subunit cDNA⁵ and encoding the sequence roughly spanning transmembrane regions 1–3. Probe-identified cloned cDNAs were sequenced and new α -subunit-encoding cDNAs used to isolate their full-length versions from the same libraries. The $\alpha 6$ subunit cDNA was one of several sequences identified in this manner. Both the rat and bovine full-length $\alpha 6$ cDNAs were inserted into the vector pCIS2 for eukaryotic expression^{6,30}.

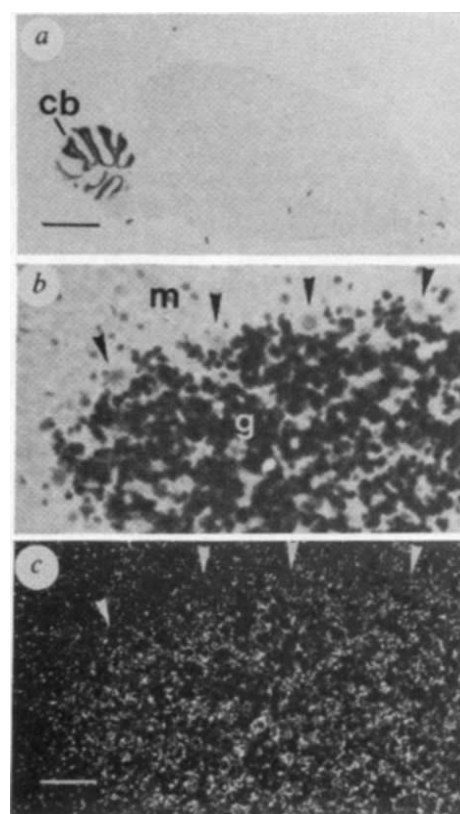


FIG. 2 Expression of α -subunit mRNA in the granule cell layer of rat cerebellum. *a*, Film autoradiogram of a sagittal rat brain section hybridized with a ³⁵S-labelled oligonucleotide complementary to the rat GABA_A receptor $\alpha 6$ subunit mRNA. *b*, High power bright-field photomicrograph of Nissl-stained cerebellum. *c*, High power dark-field photomicrograph of the same area. *d*, Grain clusters in the emulsion mark the location of the $\alpha 6$ mRNA-expressing granule cells. Arrowheads, Purkinje cells in *b* and *c*, which are not decorated by grain clusters. Abbreviations: cb, cerebellum; m, molecular layer; g, granule cells. Scale bars: *a*, 5 mm; *c*, 100 μ m. METHODS. The oligonucleotide 5' TCGACAGTACTGCTCACTTCCATTGTATCTTTTGAAAGGA 3' complementary to the coding sequence for $\alpha 6$ subunit residues 422–434 was labelled by 3' extension using terminal transferase (BRL) and [³⁵S]dATP (NEN; 1,200 Ci mmol⁻¹) to an average tail-length of 10 residues. Frozen, paraformaldehyde-fixed sagittal rat brain sections were hybridized for 24 h at 30 °C in 50 μ l hybridization solution containing 200,000 c.p.m. of the probe, 14 mM β -mercaptoethanol, 0.6 M NaCl and 50% formamide. Sections were washed in 0.5 $\times$ SSC buffer at 55 °C and dehydrated in ethanol. After exposure for 3 days to Kodak XAR5 film, the sections were dipped in Kodak NTB2 emulsion diluted 1:1 in water, and exposed for 24 days. After developing, sections were counterstained with cresyl violet.

concentrations that compete effectively at receptors containing other α -variants^{11,12}. Furthermore, the β -carbolines, β -CCM and DMCM were highly ineffective in displacing [³H]Ro15-4513 binding, having 100–4,000-fold higher K_i values than at the recombinant GABA_A-benzodiazepine type I receptors containing the $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits^{11,12}. Although disparate in their benzodiazepine binding-site properties, receptors containing either $\alpha 1$ or $\alpha 6$ subunits displayed comparable affinities for [³H]muscimol.

We raised antisera (antisera $\alpha C6$) against the C-terminal dodecapeptide of the $\alpha 6$ subunits to characterize the cognate

TABLE 1 Pharmacology of GABA_A receptors

(a) Compounds	Receptors	
	$\alpha 6\beta 2\gamma 2$	$\alpha 1\beta 2\gamma 2$
[³ H]Ro15-4513	5.4 ± 0.4*	15 ± 4*
Ro15-4513	5.1 ± 1.8	ND
Ro15-1788	90 ± 20	0.5 ± 0.2
DMCM	210 ± 50	5 ± 2
β -CCM	2,050 ± 20†	0.8 ± 0.1
2-oxo-quazepam	>10,000	20 ± 3
Diazepam	>10,000	16 ± 1
CL 218-872	>10,000	130 ± 40
Clonazepam	>10,000	1.3 ± 0.04
Flunitrazepam	>10,000	2 ± 0.3
Triazolam	>10,000	ND
Ro5-4864	>10,000	ND
[³ H]muscimol	5 ± 0.5*	7 ± 2*

(b)	[³ H]Ro15-4513	Ro15-1788	[³ H]muscimol
Precipitate	9 ± 1*	51 ± 10	6 ± 3*
Supernatant	6 ± 1*	0.6 ± 0.1	16 ± 8*

(a) Pharmacology of recombinant GABA_A-benzodiazepine receptors. Binding affinities in nM of various benzodiazepines and β -carbolines to membranes of human embryonic kidney 293 cells³⁰ expressing receptors assembled from $\alpha 6$, $\beta 2$ and $\gamma 2$ subunits, and from $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits. K_i values for the latter receptors are from ref. 12. K_i values were calculated according to the Cheng–Prusoff equation. ND, not determined. Methods were as described previously^{6,12}. (b) Pharmacology of cerebellar $\alpha C6$ -immunoprecipitated GABA_A receptors. The table lists the dissociation constant K_d ± s.e.m. values (nM) of four ([³H]Ro15-4513) and three ([³H]muscimol) experiments. The K_i ± s.e.m. values (nM) of Ro15-1788 were calculated from three experiments, using at least six data points in duplicate with Ro15-1788 concentrations of 1 nM–10 μ M and [³H]Ro15-4513 at 6 nM. Data were determined in solubilized rat cerebellar membranes after immunoprecipitation with $\alpha C6$ antisera. These antisera did not cross-react with any other GABA_A receptor α -subunit, as assayed by 293 cell expression²⁸, did not affect [³H]Ro15-1788 binding in these membranes and precipitated 60% of the diazepam-insensitive [³H]Ro15-4513 binding sites. [³H]flunitrazepam binding levels were at the detection limit in the immunoprecipitate at concentrations up to 50 nM. Cerebella from male Sprague–Dawley rats were homogenized in isotonic buffer. Crude membranes were prepared by differential centrifugation (5 min, 1,000g; 10 min, 100,000g) and were washed successively in hypotonic and isotonic buffer. The washed membrane pellet was solubilized in 2% CHAPS(3-[(3-cholamidopropyl)dimethyl-ammonio]1-propanesulphonate) containing buffer, solubilized membranes were diluted 10-fold into buffer containing 0.5% Triton X-100 and centrifuged (1 h at 100,000g). For immunoprecipitation, the supernatant was incubated overnight with ammonium sulphate-precipitated antiserum (150 μ g ml⁻¹ of supernatant) and subsequently incubated in the presence of washed *Staphylococcus aureus* (Calbiochem) for 2 h. Following centrifugation (1.5 min, 13,000g) supernatant and pellet (washed and resuspended in buffer containing 0.5% Triton X-100) were used for ligand binding. Tritiated ligands were added in final concentrations of 1–30 nM [³H]Ro15-4513 and 2–60 nM [³H]muscimol, respectively. Non-specific binding was defined by 10 μ M Ro15-1788 and 100 μ M GABA, respectively. The volume was increased to 500 μ l and final concentrations for CHAPS and Triton X-100 were 0.01% and 0.15%, respectively. After 1 h on ice, the binding was terminated by rapid filtration on glass fibre membranes (Schleicher and Schuell, number 34) pre-soaked in 0.3% polyethyleneimine, followed by two washes in 5 ml of 10 mM Tris–HCl buffer, pH 7.5.

* K_d ± s.e.m. values, all other values are K_i ± s.e.m. values.

† Measurement obtained using the bovine $\alpha 6$ subunit.

cerebellar receptor in the rat. As α -subunits differ in their C-terminal sequences, antisera generated against the C-terminal peptide of a particular α -variant will recognize the subunit on western blots and may immunoprecipitate GABA_A receptor complexes containing that subunit^{15,20}. The $\alpha C6$ antisera reacted with a band corresponding to a relative molecular mass of 57,000 (M_r 57K) on a western blot of rat cerebellar tissue (Fig. 3a). A band corresponding to a similar size was revealed by autoradiography of a gel-resolved $\alpha C6$ immunoprecipitate of [³H]Ro15-4513-photolabelled cerebellar membranes (Fig. 3b). The labelling of this band was not affected by diazepam but was prevented in the presence of Ro15-1788, indicating that the $\alpha 6$ subunit is indeed part of the Ro15-4513-selective cerebellar receptor subtype^{14,16,17}. A second, prominently labelled band corresponding to an M_r of ~51K was present in the solubilized membranes and in the supernatant after immunoprecipitation by the $\alpha C6$ antisera (Fig. 3b). This band, not labelled in the presence of either diazepam or Ro15-1788, could be precipitated by antisera specific for the $\alpha 1$ subunit^{15,20}.

Maximal concentrations of the $\alpha C6$ antisera precipitated 15–25% of all [³H]muscimol and [³H]Ro15-4513 binding sites in solubilized cerebellar membranes but the antisera specific for the $\alpha 1$ subunit precipitated ~70% of these binding sites, probably reflecting the preponderance of GABA_A-benzodiazepine type I receptors in the cerebellum^{21,22}. Both the $\alpha C6$ -precipitated and unprecipitated sites displayed similar binding affinities for [³H]muscimol and [³H]Ro15-4513 but the K_i values for Ro15-1788 displacement of [³H]Ro15-4513 binding differed by two orders of magnitude. Consistent with the immunological results, the Ro15-1788 displacement curve of [³H]Ro15-4513 binding in total cerebellar membranes was best fitted by K_i values of 0.78 ± 0.04 nM and 92 ± 11 nM for a high and a low affinity site, respectively, and a relative abundance of 20% for the low affinity site.

Comparable pharmacological characteristics were observed for the recombinant receptors containing $\alpha 1$ or $\alpha 6$ subunits (Table 1); hence, the cerebellar Ro15-4513-selective GABA_A receptor subtype probably contains $\alpha 6$, $\beta 2$ and $\gamma 2$ subunits. Such a composition is consistent with the simultaneous expression of these subunits in the cerebellar granule cells^{7,13,23}, although other subunit combinations with $\alpha 6$ might result in a similar pharmacology. A pharmacologically different class of GABA_A receptors containing an $\alpha 6$ subunit seems to exist, as roughly 35% of the [³H]Ro15-4513 binding sites that were immunoprecipitated by $\alpha C6$ antiserum were sensitive to diazepam (K_i = 15 ± 8 nM).

These results demonstrate the existence of a novel GABA_A receptor subtype expressed by cerebellar granule cells. Our data address the molecular composition of this subtype and describe the primary structure of a new α -subunit variant. The functional signature of this subunit identifies a unique benzodiazepine recognition site, as the only benzodiazepine receptor ligands that bind to it are either antagonists (Ro15-1788) or inverse agonists (Ro15-4513, DMCM). This site differs considerably in its pharmacological properties from GABA_A-benzodiazepine type I and type II receptors, the latter constituting a heterogeneous population^{11,12}. Hence, a new classification of benzodiazepine receptors is required and should be based on molecular composition.

A cerebellar site with similar properties has been described^{14,16} and, because of its unusual pharmacological profile, was viewed as a nonspecific binding site¹⁴. But the properties of the new GABA_A receptor offer a molecular explanation for several biochemical and behavioural observations. Notably, the vastly reduced affinities for many benzodiazepines and β -carbolines make this subtype a good candidate for the cerebellar GABA_A receptor lacking a benzodiazepine site, whose existence had been proposed on the basis of the disparate localization of [³H]muscimol and [³H]flunitrazepam binding (as determined by receptor autoradiography)^{24–26}.

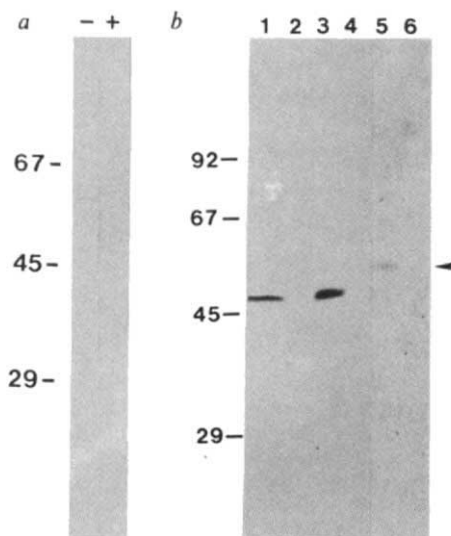


FIG. 3 Analysis of $\alpha 6$ subunit-containing GABA_A receptors in rat cerebellar membranes. *a*, Immunoblot of SDS-polyacrylamide gel-resolved rat cerebellar membranes stained with the $\alpha 6$ -subunit-specific $\alpha C6$ antiserum in the absence (–) and presence (+) of the C-terminal dodecapeptide. *b*, Autoradiograph of gel-resolved rat cerebellar membranes labelled irreversibly by [3 H]Ro15-4513 in the presence of 10 μ M Ro15-1788 (lanes 2, 6), 10 μ M diazepam (lanes 4, 5) or in the absence of any inhibitor (lanes 1, 3). Lanes 1 and 2, supernatant after immunoprecipitation by $\alpha C6$; lanes 3 and 4, material immunoprecipitated by an $\alpha 1$ -specific antibody prepared as described previously^{15,20}; lanes 5 and 6, material immunoprecipitated by $\alpha C6$. The bands in *a*, lane – and *b*, lane 5 that react with the $\alpha C6$ antisera show the same electrophoretic mobilities (calculated $M_r \sim 57.9$ K and 56.5K, respectively) within the experimental errors caused by aligning unlabelled and 14 C-labelled protein standards (Amersham; indicated on the left of *a*, *b*). **METHODS.** Membranes were prepared as in Table 1. For the immunoblots, pelleted membranes were solubilized in SDS sample buffer, proteins resolved on a 12% polyacrylamide SDS gel and electroblotted on polyvinylidene difluoride (PVDF) membranes²⁷. Immunostaining used 1% BSA in phosphate buffered saline with 0.05% Tween-20 (Tween-PBS) as a blocking agent (1 h at 37 °C). The primary antibody (3 μ g ml^{–1}) was added overnight at 4 °C to the PVDF membranes in 1% BSA/Tween-PBS in the presence or absence of the C-terminal dodecapeptide (10 μ g ml^{–1}). The membranes were incubated in a peroxidase-anti-peroxidase (PAP) system (Dianova) at antibody dilutions of 1:1,000 for the bridge antibody and the PAP complex, using extensive washes between incubations. The bands were visualized using 3,3'-diaminobenzidine (50 μ g ml^{–1}) and hydrogen peroxide (0.03%) as substrate. Autoradiography data were obtained after photolabelling (10 min at 364 nm) cerebellar membranes using 10 nM [3 H]Ro15-4513 (ref. 14) in the presence or absence of 10 μ M inhibitor. Unreacted [3 H]Ro15-4513 was displaced by 10 μ M Ro15-1788 (20 min, 4 °C). After three washes of the pellet in 50 mM potassium phosphate buffer, pH 7.0, and a wash in 10 mM Tris-HCl buffer, pH 7.5, the pellets (20 min at 23,000g) were solubilized and immunoprecipitated as in Table 1. Supernatants and pellets were subjected to SDS-PAGE and western blotting as described above. Autoradiography used En 3 Hance spray. The autoradiogram was exposed for 40 days.

Most importantly, the newly characterized GABA_A receptor seems to be functionally involved in mediating the alcohol-induced impairment in motor performance that can be antagonized at the same receptor by Ro15-4513. Elegant evidence for this idea derives from the biochemical analysis of rat lines differentially affected in their sensitivity to ethanol, benzodiazepines and barbiturates. In these animals, [3 H]Ro15-4513 labelling of a cerebellar protein of $M_r \sim 57$ K shows differential sensitivity to diazepam¹⁷. Our molecular and immunological analyses are consistent with the proposal that this protein represents the $\alpha 6$ subunit expressed by cerebellar granule cells. The cloned cDNA encoding this subunit should constitute an excellent probe for the analysis of the homologous protein in the rat lines. The correct recombinant re-creation of the new GABA_A receptor subtype may allow the development of new

ligands for modulating channel activity at this neurophysiologically unique receptor subtype. □

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Novel mechanism of voltage-dependent gating in L-type calcium channels

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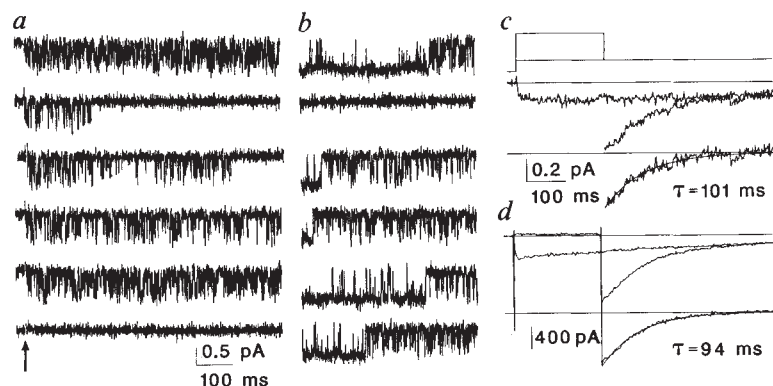
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ACTIVATION of voltage-dependent calcium channels by membrane depolarization triggers a variety of key cellular responses, such as contraction in heart and smooth muscle and exocytotic secretion in endocrine and nerve cells. Modulation of calcium channel gating is believed to be the mechanism by which several neurotransmitters, hormones and therapeutic agents mediate their effects on cell function. Here we describe a novel type of voltage-dependent equilibrium between different gating patterns of dihydropyridine-sensitive (L-type) cardiac Ca²⁺ channels. Strong depolarizations drive the channel from its normal gating pattern into a mode of gating characterized by long openings and high open probability^{1,2}. The rate constants for conversions between gating modes, estimated from single channel recordings, are much slower than normal channel opening and closing rates, but the equilibrium between modes is almost as steeply voltage-dependent as channel activation and deactivation at more negative potentials. This new mechanism of voltage-dependent gating can explain previous reports of activity-dependent Ca²⁺ channel potentiation in cardiac³ and other cells^{2,4,5} and forms a potent mechanism by which Ca²⁺ uptake into cells could be regulated.

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FIG. 1 Prepulses to positive potentials induce a distinct gating pattern in single L-type Ca^{2+} channels. *a, b*, Cell-attached recording from an adult rat ventricular myocyte with 110 mM Ba^{2+} as charge carrier. The patch contained a single L-type Ca^{2+} channel. *a*, Unitary currents in response to step depolarizations from -70 mV to a test potential of $+10$ mV. Inward currents (channel openings) shown as downward deflections. The depolarization was applied at the time of the arrow in the last trace. *b*, Current traces obtained at the same test potential as in *a*, but following an initial depolarization for 224 ms from the holding potential to a prepulse potential of $+110$ mV. Currents are shown immediately after repolarization to the test potential. Voltage protocols with and without prepulse were alternated and currents shown are consecutive (from 1. trace in *a* to 1. trace in *b*, and so on), but are separated into two columns for clarity. Interval between successive depolarizations was 4 s. *c*, Average currents from same patch as in *a* and *b*. Currents with and without prepulse were averaged separately and are superimposed. The difference between the two currents is shown in the trace below the averages and is fitted to a decaying exponential with a time constant of 101 ms. Holding potential -70 mV, test potential $+10$ mV, prepulse potential $+110$ mV. *d*, Whole-cell recording from an isolated chick atrial myocyte. 20 mM Ba^{2+} is the charge carrier. Similar voltage protocol as in single channel experiment, but with more negative test- (-10 mV) and prepulse- ($+80$ mV) potential to compensate for the shift resulting from the lower concentration of divalent ions in the whole-cell recording. Current traces elicited by depolarizations with and without prepulse are superimposed, and the difference, fitted by a decaying exponential with a time constant of 94 ms is shown underneath.



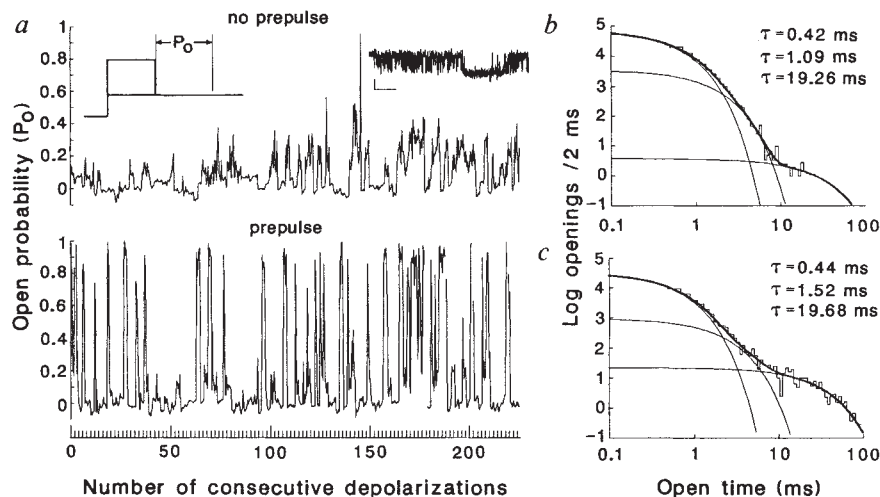
METHODS. Single cardiac myocytes were prepared by a standard enzymatic dissociation¹. Cell-attached recordings²⁴ were obtained with the following solutions: Pipette (external solution, in mM): 110 BaCl_2 , 10 HEPES, pH 7.4. Bath: 145 potassium aspartate to zero membrane potential outside the patch, 5 EGTA, 10 HEPES, pH 7.4. Solutions for whole-cell recordings contained: Pipette (internal solution, in mM): 135 CsCl, 5 BAPTA, 10 HEPES, 4 ATP, 5 MgCl_2 , pH 7.4. Bath: 20 BaCl_2 , 135 TEA-Cl, 10 HEPES, pH 7.4. Special care was taken to keep all solutions and recording chambers absolutely drug-free. Currents were recorded by a LIST-EPC7 or Dagan 3900 patch clamp amplifier and stored and analysed on a DEC 11/73 laboratory computer. Signals were sampled at 5 kHz and filtered at 1 kHz (8-pole Bessel filter, -3 dB). Linear leak and capacitance currents were subtracted digitally. All recordings were at room temperature (22 – 25°C).

The existence and possible importance of a distinct gating mode of L-type Ca^{2+} channels, characterized by long openings and high open probabilities (p_o) (ref. 1), has remained controversial^{6,7}. To resolve this issue, and in an attempt to understand the mechanisms underlying transitions between different gating modes, we tested the hypothesis that the equilibrium between gating modes was voltage dependent, a possibility which was suggested by the known potentiation of Ca^{2+} currents by strong depolarizations or repetitive action potentials^{2–4}. We chose cardiac cells, because of the ease with which L-type Ca^{2+} channels can be unambiguously identified in this preparation^{8,9}. Figure 1 shows unitary current recordings from a membrane patch which contained a single L-type Ca^{2+} channel. Normal depolarizing pulses were alternated with pulses in which the test potential ($+10$ mV) was preceded by a prepulse to $+110$ mV for 224 ms. Normal depolarizations elicited the familiar rapid gating pattern of L-type Ca^{2+} channels (Fig. 1*a*), with brief

openings grouped in bursts^{1,6,10}. In contrast, channel activity immediately following the prepulse (Fig. 1*b*) very often consisted of greatly prolonged openings, separated by brief closings, resulting in a much higher open probability. During the remaining 500 ms of the test pulse at $+10$ mV, this prepulse-induced gating pattern abruptly returned to the brief-opening pattern observed in sweeps without prepulse. As expected, a comparison of the averaged currents with or without prepulse (Fig. 1*c*) showed a much larger inward current following the prepulse. The extra current induced by the prepulse decayed exponentially with a time constant of ~ 100 ms at $+10$ mV with 110 mM Ba^{2+} as the charge carrier (difference trace in Fig. 1*c*). The response of the single channel illustrated in Fig. 1*a–c* is representative of all L-type channels in a cardiac cell, as shown by the whole-cell Ba^{2+} currents obtained under similar experimental conditions from an intact cardiac cell (Fig. 1*d*).

Prepulse-induced changes in gating pattern represent a robust

FIG. 2 Stationarity of the response to prepulses, and comparison of open time distributions with and without prepulse. *a*, Time plot of open probability p_o measured in a window of 240 ms following the end of the prepulse or during the same time window in sweeps with no prepulse. Each point represents p_o measured during a 12-ms time bin within that time window. Same patch and voltage protocol as in Fig. 1 *a–c*. Depolarizations with and without prepulse were delivered alternately at 4-s intervals, but are shown separately in the upper and lower part respectively. Horizontal axis gives sweep numbers, each small unit represents the duration of the 240-ms time window in one sweep. Total duration of experiment shown was 15 min. Inset, single channel trace with episode of the high- p_o mode without a prepulse. Calibration bar for the inset, 100 ms, 0.5 pA. *b, c*, Log-log plot of the open time distributions obtained from all sweeps without prepulse (*b*) or from 550-ms segments following the prepulse (*c*). The dark solid line is the best-fitting sum of three decaying exponential components. Each exponential component is shown as a light solid line. The best fit was determined by the method of maximum likelihood and a minimum of three components was indicated by the maximum likelihood ratio test^{25,26}. Log binning and fitting of the binned distribution was done as described in refs



26 and 27. The time constants for the best fit are indicated above each plot. The fastest time constant is poorly resolved. After the prepulse the component with the longest time constant is greatly enlarged, with little change in the other components.

response which can be elicited consistently for the entire duration of a single channel recording (Fig. 2a). There was a very strong correlation between prepulses and induction of the high- p_o gating pattern, and this phenomenon was stationary over many minutes. By contrast, in sweeps without prepulses, episodes with long-lasting openings were rarely observed (see Fig. 2a, inset). The reproducible induction of the high- p_o gating pattern made it possible to study the kinetics of the transitions within this mode. Fits of open time distributions obtained from sweeps with or without prepulse each required a minimum of three exponential components. The two distributions shown in Fig. 2b, c differ mainly in the relative amplitudes of the longest component, which accounts for 3% and 0.2% of all openings in sweeps with or without prepulse respectively. Thus, positive potentials seem to shift the equilibrium between open states toward the long-lasting one.

For the following analysis, we considered a simplified kinetic scheme, in which activity with brief openings and activity with long openings are treated as two distinct kinetic modes¹ and the two modes are connected by single forward (k_f , entry into the high- p_o mode) and backward (k_b) first-order rate constants (see Methods in Fig. 3 legend).

We measured the transition rates between modes by repeating prepulse experiments at different prepulse and test potentials. To obtain estimates that would allow more direct conclusions about the equilibrium between modes in the physiological voltage range, we minimized the unavoidable shift towards positive voltages induced by high concentrations of divalent cations by carrying out single channel recordings in 10 mM Ba^{2+} (Fig. 3a). We estimated the rate constant for the disappearance of the high- p_o mode (k_b) by measuring the time constant of the decay of the prepulse-induced current from averaged currents in patches containing from one to five channels. Figure 3c shows a plot of k_b as a function of voltage, obtained from one patch: k_b decreases roughly exponentially with voltage, with an e -fold change per 11.5 mV. In a total of seven experiments with 10 mM Ba^{2+} , the average voltage dependence was e -fold per 13.39 ± 1.05 mV (mean $\pm$ s.e.m.).

We assumed that the forward transition into this mode could also be described by a single rate constant, k_f , and estimated its value by counting the fraction of current traces in which a prepulse had induced the appearance of the high- p_o mode (see Methods in Fig. 3 legend). An experiment that gave values for k_f at four potentials is shown in Fig. 3d. As expected, k_f increases with voltage, but its voltage dependence is much less pronounced than that of k_b . In three experiments, k_f changed by e -fold for every 41.2 ± 2.9 mV (mean $\pm$ s.e.m.).

Our values for k_f and k_b should be able to predict correctly the dependence of the prepulse-induced current on prepulse length. This is indeed the case, as shown in Fig. 3f, where data points for two prepulse potentials and various prepulse durations are compared with the predicted time course computed with the values for k_f and k_b derived as described in the legend to Fig. 3.

In Fig. 4, we compare the voltage dependence of the equilibrium between gating modes with the voltage dependence of channel activation. Examples of current traces obtained with 10 mM Ba^{2+} as charge carrier are shown in Fig. 4a, and the data points are fitted by a Boltzman distribution in Fig. 4b (left-hand smooth curve). The steady-state distribution between the two modes of gating was calculated from our mean values of k_f and k_b , obtained at the same ionic conditions, and is shown as the right-hand smooth curve in Fig. 4b. The midpoint of the steady-state activation curve is approximately 25 mV more negative than that for the equilibrium between modes. The voltage dependences of the two curves are rather similar, with a maximal steepness corresponding to an equivalent charge of 3.2 for the activation curve, and 2.6 for the equilibrium between modes. The relative position of the voltage-dependent parameters obtained with 10 mM Ba^{2+} should be no more than 5–10 mV

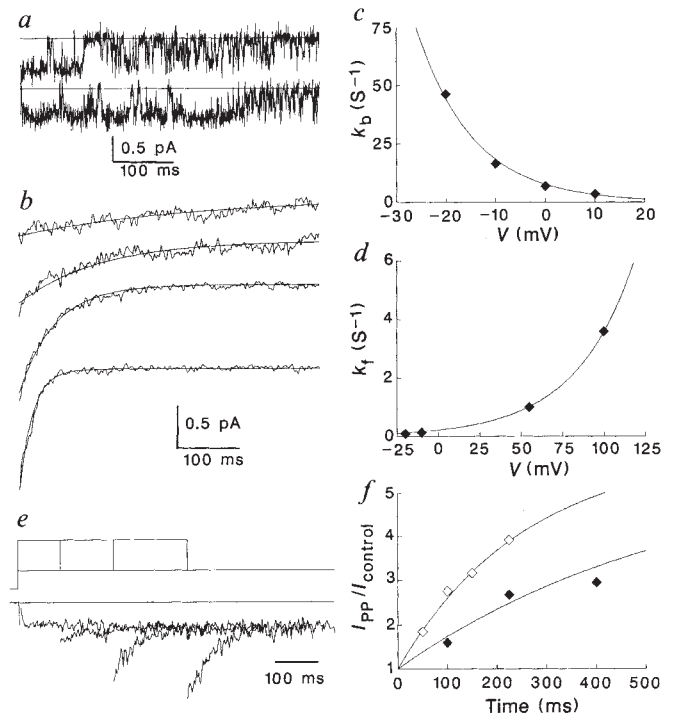


FIG. 3 Kinetics of the transitions into and out of the high- p_o mode. All data from single channel recordings made on rat ventricular myocytes. Experimental conditions as in Figs 1 and 2, except that the recording pipette contained (in mM): 10 BaCl_2 , 135 NaCl , 10 HEPES , pH 7.5. a, Two current traces from a patch containing a single channel. Test potential -10 mV, immediately following a 224-ms prepulse to $+100$ mV. b, Prepulse-induced currents at different test potentials. Traces are averaged single channel currents from a patch which contained at least five channels. Holding potential -60 mV, prepulse potential $+100$ mV, test potential from top to bottom trace: $+10$, 0 , -10 and -20 mV, respectively. Solid lines are single exponential fits. c, Voltage dependence of k_b ; k_b is the rate constant for exit from the high- p_o mode (backward rate constant) obtained as the reciprocal of the time constants derived from the currents in b. The smooth line is an exponential fit of the data points by $k_b = 7.69 \times \exp(-2.25 \times VF/RT)$. d, Voltage dependence of k_f ; k_f is the rate constant leading to the high- p_o mode (forward rate constant). Values at four voltages from a patch containing a single channel. The smooth line is $k_f = 0.22 \times \exp(0.72 \times VF/RT)$. e, f, Dependence of the prepulse-induced current on prepulse length. e, Average current traces from sweeps with various prepulse lengths are superimposed. Prepulse voltage, 50 mV; test potential, -20 mV. f, Ratio of the peak current after the prepulse to the steady current at the end of the test depolarization for various durations of prepulses. $\diamond$, Prepulse potential $+90$ mV; $\blacklozenge$, prepulse potential $+50$ mV; solid lines, expected dependence of the prepulse-induced current on prepulse length, calculated from the equation below with the values of k_f and k_b derived from the same experiment. The two curves for the two prepulse potentials are scaled by the same factor.

METHODS. Combining several individual states into one 'mode' of gating is justifiable if transitions between states in a mode are much faster than transitions between modes. The predominance of single-exponential relaxations underlies the choice of single forward (k_f) and backward (k_b) rate constants for the transitions between modes. k_b is obtained as the reciprocal of the time constant of the decay of the prepulse-induced averaged current. This is justified as long as $k_b \gg k_f$, a condition which is valid at normal test potentials, where channels in the high- p_o mode return to the low- p_o with very little chance to enter the high- p_o mode again during the remainder of the test pulse. To estimate k_f from single channel recordings, we counted the fraction of current traces in the long opening mode immediately following the prepulse, excluding sweeps in which no channel activity was recorded. This fraction, denoted as p_n , is the probability that the channel is in the high- p_o mode at the end of the prepulse and is given by $p_n = [k_f / (k_f + k_b)] \times \{1 - \exp[-(k_f + k_b) \times t_{pp}]\}$, where t_{pp} is the prepulse duration. We used the voltage dependence of k_b to extrapolate the value of k_b to the prepulse voltages and thus were able to obtain k_f from the equation. At negative voltages we counted the fraction of the total 'active' time a single channel spent in the high- p_o mode (without prepulses). This fraction $f_n = (k_f / (k_f + k_b))$ also yielded a value for k_f .

more positive than those expected with physiological concentrations of divalent ions. We found that the equilibrium between modes in 110 mM Ba^{2+} was shifted to ~ 25 mV more positive potentials (data not shown). This shift was entirely due to a shift in the values of k_b , whereas k_f was insensitive to the Ba^{2+} concentration.

To judge whether the long opening mode might contribute significant current during physiological channel activity, one has to consider not only the steady-state distribution between modes, but also the effective time constant of the approach to the steady state. The predicted voltage dependence of this time constant ($\tau_m = 1/(k_f + k_b)$) is shown as the bell-shaped curve in Fig. 4b. It increases from around 50–200 ms between -20 and 0 mV and reaches a peak of around 600 ms at $+30$ mV. Thus it is unlikely that long openings contribute significantly to single short action potentials typical of nerve and muscle. The long duration of cardiac action potentials and the voltage range of the cardiac action potential plateau, however, are such that transitions between modes should be fast enough to shift channels to the high- p_o mode¹¹. Although at potentials negative to 0 mV only a small fraction of channels would be expected in the high- p_o mode, that gating would still contribute significantly to the overall current because of the much longer open times and higher p_o in that mode. A progressive shift towards the high- p_o gating mode would also be expected for trains of rapidly delivered short action potentials, such as those used to elicit

long-term potentiation¹². At negative voltages τ_m becomes very short (Fig. 4b), and so we would expect progressive potentiation of L-type Ca^{2+} currents to occur during action potentials or voltage clamp depolarizations only if the depolarizations are separated by no more than 10–20 ms at resting potentials negative to -40 mV.

The present report should lay to rest the controversy about the existence of several gating modes in L-type Ca^{2+} channels^{6,7}. Clearly, strong depolarization do more than favour transitions into just one additional open state². Rather, positive potentials drive the channel into a different set of states, which we designate as a gating mode because at least at positive potentials transitions between the states in each mode are much more rapid than transitions between modes. But because of the voltage dependence of k_b , near the threshold of channel activation, where most single channel studies in Ca^{2+} channels are performed, the lifetime of the high- p_o mode exceeds the duration of individual long events only by a factor of 2–5, such that the moding pattern is much less obvious.

It is clear that shifts along the voltage axis of the voltage-dependent equilibrium between modes would constitute a very potent mechanism for modulation of L-type Ca^{2+} channels. The action of dihydropyridine Ca^{2+} channel agonists can be interpreted in this way. A more rapid k_f and a slower k_b would result in single channel currents at normal test potentials similar to those observed experimentally^{1,13}. Physically, however, drug-bound channels must still be represented as yet a different set of states, even if their functional properties cannot easily be distinguished from drug-free high- p_o states.

Although in initial reports^{14,15} β -adrenergic agonists were not found to promote long-lasting openings, Yue *et al.*¹⁶ have recently shown that isoproterenol can significantly enhance the appearance of the high- p_o mode in cardiac cells. Thus, channel phosphorylation¹⁷ may also shift the equilibrium between gating modes. Even under our drug-free cell-attached recording conditions we have occasionally observed the appearance of high- p_o activity lasting for seconds to minutes which was not clearly correlated with strong depolarizations. This further suggests that other mechanisms, in addition to membrane depolarization, can perturb the equilibrium between gating modes.

The demonstration of a second voltage-dependent equilibrium of channel gating suggests that the S4 region, common to all voltage-sensitive ion channels (for review, see ref. 18), may not be the only voltage sensor, or may transduce the change in voltage in more than one conformational change. Consistent with our data, Bean and Rios¹⁹ have recently reported an extra component of cardiac Ca^{2+} channel activation at positive potentials.

Finally, it must be noted that the lumping together of two distinct gating patterns into modes, and the assignment of single backward and forward rate constants for the transitions between modes, must remain a considerable oversimplification of the complex gating pattern of L-type Ca^{2+} channels. We and others^{17,20} have noticed additional gating modes that cannot easily be classified into the two main patterns presented here, and occasionally more than one exponential was required to fit the decay of the prepulse-induced currents. For a full understanding of the kinetic processes involved, it would be desirable to establish not only sets of kinetic states as modes, but also the exact connectivity between all the individual states. Gating modes have recently been described in other voltage-activated ion channels^{21–23}. It will be important to find out whether transitions between modes in these channels are also voltage-sensitive. □

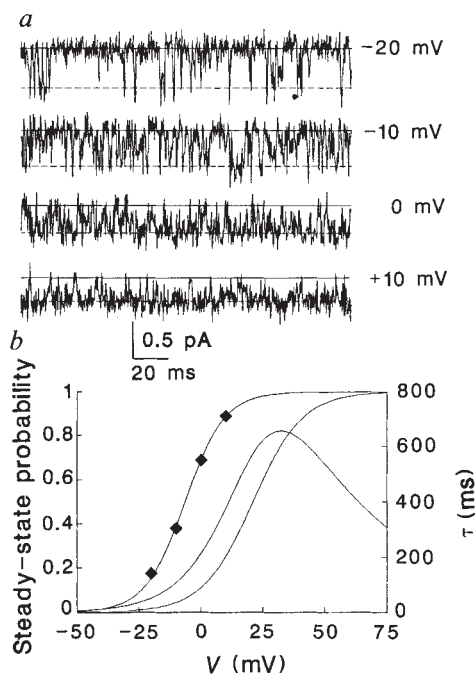


FIG. 4 Comparison of steady-state activation curve and the equilibrium between modes. Single channel recordings with 10 mM Ba^{2+} as charge carrier. Experimental conditions as in Fig. 3. *a*, Sample traces of the short-opening gating mode at the test potentials indicated to the right of each trace. Note expanded timescale compared with Figs 1–3. In *b*, $\blacklozenge$ represent steady-state open probability, p_o , of the short-opening mode; p_o was measured from segments of current traces such as those shown in *a* by integrating the area between the closed and open level and dividing it by the elementary current. Sweeps with no openings or with transitions to absorbing closed states (see for example, second trace in Fig. 1*a*) were not included. Thus, the values of p_o reflect the voltage-dependent equilibrium between short-lived open and closed states during the predominant gating pattern. The data points are fitted by a Boltzmann distribution of the form $p_o = \{1 + \exp[(V - V_{1/2}) \times zF/RT]\}^{-1}$, with $z = 3.21$ and $V_{1/2} = -6.5$ mV. The right-hand sigmoidal curve is the steady-state distribution between modes, calculated with $k_f = 0.524 \times \exp(V \times 0.624 \times F/RT)$ and $k_b = 4.16 \times \exp(-V \times 1.92 \times F/RT)$. The bell-shaped curve represents the time constant $\tau_m = 1/(k_f + k_b)$ for the approach to equilibrium of the distribution between modes, and its scale is given on the right.

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Cycling of cell-surface MHC glycoproteins through primaquine-sensitive intracellular compartments

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CLASS II major histocompatibility complex (MHC) glycoproteins associate with peptides derived from material endocytosed by antigen-presenting cells and processed along the endocytotic pathway^{1–3}. No consensus exists as to what extent class II molecules themselves are endocytosed and it is not known whether endocytosed MHC class II molecules can be recycled again to the cell surface—an itinerary which might allow a single cell-surface MHC molecule to associate with different peptides during its lifetime. We now show by using new cleavable labelling reagents⁴ that class II and class I MHC on B lymphoblastoid cells are continually endocytosed and recycled to the cell surface. The intracellular pool size is normally kept small by efficient recycling, but in the presence of primaquine the rate of recycling is slowed, thereby increasing the size of this pool substantially. On removal of the amine, the intracellular population recycles rapidly to restore the original distribution. These results reveal a cycle that might explain the rapid binding and turnover of some peptide/class II MHC complexes^{5,6} and the exchange of pre-existing for new peptides⁷ observed in living cells.

Although several studies have shown that cell-surface MHC glycoproteins can be internalized^{8–11}, other work suggests that they are not^{12,13}, or that the phenomenon is specific for cell type or MHC class¹⁴. In no case has it been possible to show directly that internalized MHC is recycled to the cell surface—a prerequisite for endocytosis of MHC to be relevant to peptide exchange. We have used a new class of cell-surface iodinating reagents described by Bretscher^{4,17} to address this question. These reagents incorporate an iodinated tyrosine residue linked by means of a disulphide bridge to an *N*-hydroxysuccinimide ester which can react with cell-surface amino groups. The labelled portion of the reagent can therefore be cleaved from the cell surface with impermeant reducing agents such as glutathione (GSH) allowing intracellular and cell-surface populations to be distinguished⁴. The Epstein–Barr virus transformed lymphoblastoid cell line A46^{15,16} was labelled with DPSgtc (dithiopropionyl, sulphosuccinimidyl, glycyl ¹²⁵I-tyrosyl cholamine) at 0 °C, and

either held at 0 °C or incubated at 37 °C for 30 minutes. Aliquots of each cell sample were then incubated at 0 °C in GSH for 30 min to remove the radiolabel remaining on the cell surface^{4,17}. Glycoproteins were immunoprecipitated and run on SDS gels under non-reducing conditions. On cells held at 0 °C, the radiolabel associated with the cell-surface transferrin receptors, class I and class II MHC was efficiently removed with reduced GSH (Fig. 1a, tracks 2, 5 and 8). Following a 30-min incubation at 37 °C, a large fraction of the transferrin receptor became resistant to GSH treatment (Fig. 1a, track 3)⁴ whereas in the same cells, 7 ± 1.8 and 3 ± 1.1% (s.d.; *n* = 7) respectively of the labelled cell surface population of class II and class I MHC became GSH-resistant (Fig. 1, tracks 4–6, 7–9).

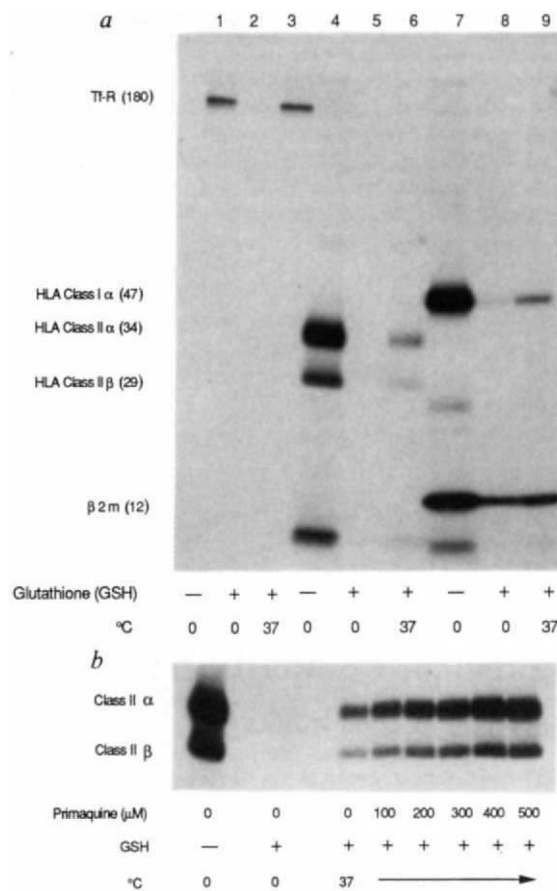
We designed two experiments to determine whether the MHC molecules entering this small intracellular pool could recycle to the cell surface. First, if continual cycling was occurring then conditions which selectively slow the return to the cell surface should increase the intracellular pool size. Second, return of this pool to the surface should be signalled by reacquisition of sensitivity to GSH.

Primaquine (Pq) slows the recycling of transferrin, asialoglycoprotein and other receptors, thereby increasing their intracellular pool size^{18,19}. In A46 cells surface labelled with DPSgtc and then incubated at 37 °C in the presence of Pq, the proportion of class II molecule that became resistant to GSH increased dramatically (Fig. 1b). Over a 30-min period at 37 °C, 23.6 ± 4.2% of cell-surface class II and 19 ± 3.6% of class I molecules (s.d.; *n* = 5) became resistant to GSH (Fig. 2). In the absence of Pq the small intracellular pool of MHC equilibrated in 3–4 min, whereas in the Pq-treated cells the now larger pool took between 10 and 15 min to equilibrate, making it possible to measure the kinetics of endocytosis. Over a 1-min period at 37 °C, 2.1 ± 1.0% of surface class II and 1.7 ± 0.4% of class I molecules were endocytosed (s.d., *n* = 5; Fig. 2), which, assuming first-order kinetics, indicates a half life on the cell surface of 33 and 41 min respectively. MHC glycoproteins labelled with DPSgtc failed to become GSH-resistant under hyperosmotic conditions that block endocytosis through coated pits^{20–22}, confirming that the assay measured endocytosis and indicating that coated pits are the entry points for MHC glycoprotein molecules, in line with earlier morphological data for class I molecules²³.

On the basis of the striking accumulation seen in the presence of Pq we suggest that MHC glycoproteins are endocytosed but are then efficiently recycled to the cell surface, thus maintaining small intracellular pools which are difficult to detect by other methods. Recycling should render labelled MHC molecules in the intracellular pool sensitive once again to GSH. Moreover, given the normal surface/intracellular distribution of 93/7 observed for class II molecules (Fig. 1) and an inward rate of 0.021 min^{−1} (Fig. 2), we would predict an outward rate of recycling of 0.28 min^{−1}, corresponding to a half life of the intracellular pool of about 2.4 min. In other words, a large fraction of the internal pool should recycle rapidly if we are correct in concluding that this is how the normally small intracellular pools are maintained. Labelled cells were incubated at 37 °C in the presence of Pq and then treated with GSH at 0 °C to remove label from molecules remaining on the cell surface. Aliquots of cells were then reincubated in the presence or absence of Pq at 37 °C to allow membrane traffic to resume and at different times were exposed to a second treatment with GSH. About 80% of intracellular class II and 90% of class I MHC molecules became sensitive to GSH treatment within 15 min, demonstrating directly that extensive recycling to the cell surface had occurred with a half life of about 2–3 min, as predicted (Fig. 3). As expected, when Pq was present recycling still occurred, but was slower following an initial lag which was required for Pq to re-enter the cells (Fig. 3). Cells reincubated at 37 °C but not exposed a second time to GSH showed no loss of label, ruling out the possibility that the label was being cleaved off within the cell (Fig. 3, track 8).

FIG. 1 Endocytosis of DPSgtc-labelled MHC glycoproteins. *a*, Lymphoblastoid cell line A46 was labelled with ^{125}I -labelled DPSgtc at 0 °C and incubated at 0 °C or 37 °C for 30 min. Aliquots were treated with reduced GSH (Sigma) as indicated, washed and lysed in Nonidet P-40. Labelled transferrin receptor, class II MHC and class I MHC molecules were recovered sequentially from the same lysates by immunoprecipitation and electrophoresed on a 5–20% gradient SDS gel. *b*, Labelled cells were incubated at 37 °C for 30 min in medium containing increasing concentrations of Pq. Cell surface radiolabel was removed with GSH and intracellular class II analysed by immunoprecipitation, gel electrophoresis and autoradiography. Gamma-counting of MHC chains excised from the dried gels showed that the efficiency of GSH reduction on cells held at 0 °C was $96 \pm 1.0\%$ and $92.5 \pm 1.4\%$ for the α - and β -chains of class II (s.d., $n=17$) and $96 \pm 0.6\%$ for class I α -chain (s.d., $n=16$) whereas only $79 \pm 2.0\%$ (s.d., $n=10$) of label incorporated into $\beta 2$ -microglobulin ($\beta 2\text{m}$) was cleaved (see *a*, track 8). High efficiency of cleavage by GSH is essential for endocytosis into small intracellular pools to be measurable. Relative molecular masses are indicated in parentheses ($M_r \times 10^{-3}$).

METHODS. DPSgtc was prepared from DTSSP (Pierce) and glycol tyrosyl cholamine chloride hydrochloride (gtc) exactly as described⁴ and iodinated ($1 \text{ mCi } ^{125}\text{I}$ per nmol gtc) just before use. The unlabelled reagent was stored in small aliquots at -70°C . A46^{15,16} cells were washed in protein-free medium, resuspended in $0.1 \text{ M Na}_2\text{HPO}_4$ and labelled at 0 °C at a density of $\sim 10^8$ – 10^9 cells per ml^{-1} in a final volume of $\sim 75 \mu\text{l}$ as described⁴. After 20 min the cells were washed in DPBS containing 10% FCS and resuspended at $5 \times 10^6 \text{ ml}^{-1}$ in RPMI medium, 20 mM HEPES buffer, pH 7.5, 10% FCS (RPMI/H/FCS). Aliquots of 5×10^6 cells were held at 0 °C or incubated at 37 °C for 30 min in the absence (*a*) or presence (*b*) of Pq as indicated. Pq was added from a 3.0 mM stock solution dissolved in RPMI-HEPES and the final pH adjusted to 7.6 before addition of 10% FCS. After washing and resuspending in DPBS/10% FCS, the cells were treated at 0 °C for 30 min with GSH at a final concentration of 46 mM (1.6×10^6 cells ml^{-1}). The excess GSH was removed by washing the cells and then resuspending them in 27 mM iodoacetamide for 10 min before lysis in 1.7% NP40 as described⁴. The lysates were cleared by microfuging (13,000g, 15 min) and then by centrifugation in the Beckman Airfuge at 100,000g for 30 min. The transferrin receptor, class I and class II MHC glycoproteins were recovered by sequential incubation with monoclonal antibodies HuLy-m9, W6/32 (Serotec) and DA6.231. Each monoclonal antibody was recovered with affinity-purified rabbit anti-mouse immunoglobulin (Serotec) and protein A Sepharose before the addition of the next monoclonal. Immune complexes were washed and eluted in nonreducing SDS sample buffer and displayed on 5–20% (*a*) or 15% (*b*) SDS gels which were dried and exposed to Fuji RX film.



MHC class II molecules remaining on the cell surface in the presence of Pq were still cycling into and out of the cell because when we labelled cells which had been pretreated with Pq for 30 min at 37 °C, endocytosis of this population into a Pq-sensitive intracellular pool still occurred (not shown). This suggests that most cell-surface class II molecules are involved in recycling and that this occurs independently of associated invariant chain as only a small fraction of cell-surface class II molecules are thought to be associated with this additional polypeptide. Endocytosis of class II MHC molecules was also observed when DA6-147 antibody, specific for DR α chain^{24,25} was used for immunoprecipitations.

These results establish that cell-surface MHC glycoproteins spontaneously cycle through Pq-sensitive, presumably acidic endosomes. Similar results were obtained on a second lymphoblastoid cell line (clone 11.3¹⁵) isolated from a different donor. Chloroquine did not block the recycling of MHC glycoproteins in B lymphoblastoid cells over the time course (30 min) of the experiments reported here; nor did the ionophore monensin, consistent with recent findings by Davis and Cresswell¹³. But monensin (10 μM), primaquine and chloroquine (300 μM) all inhibited proteolysis of endocytosed antigen to the same extent (70–80% inhibition of appearance of trichloroacetic acid-soluble material over a 2- or 4-h period)²⁶, indicating that each had a

FIG. 2 Kinetics of MHC glycoprotein endocytosis. Cells were labelled at 0 °C, washed and incubated at 37 °C in the presence (●) or absence (□) of 0.3 mM Pq for various times as described in Fig. 1. Each aliquot was treated with GSH to remove cell-surface radiolabel, blocked with iodoacetamide and MHC glycoproteins recovered by immunoprecipitation, SDS-polyacrylamide gel electrophoresis and autoradiography. Bands corresponding to the α -chains of class II (*a*) class I (*b*) molecules were excised from the dried gel and radioactivity quantitated by gamma-counting. Aliquots of cells which had not been treated with GSH or which had been exposed to GSH without previous incubation at 37 °C were electrophoresed in parallel, MHC bands excised and counted. The fraction of labelled cell-surface MHC becoming GSH-resistant is plotted after subtraction of the background resistant to GSH on cells held at 0 °C (~ 2 –3% of total).

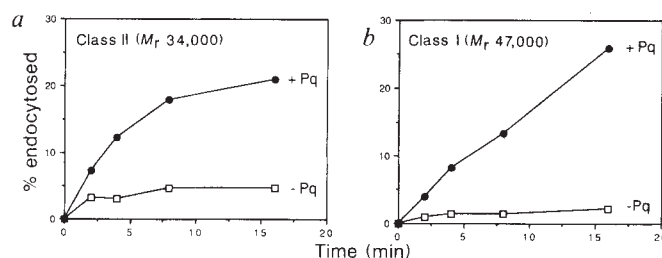
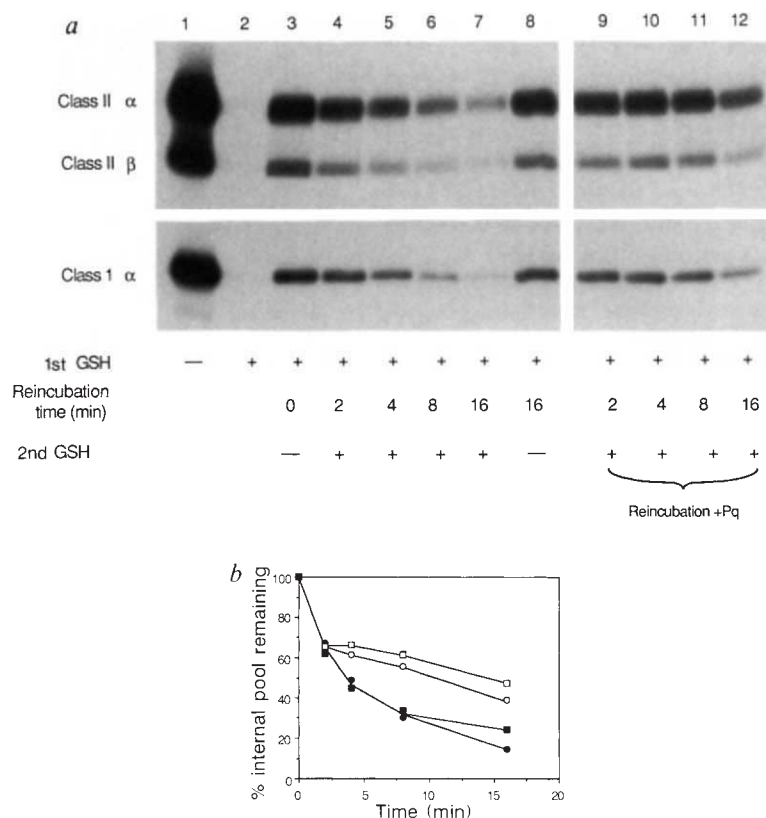


FIG. 3 Endocytosed MHC glycoproteins recycle to the cell surface. *a*, Cells were labelled and either held at 0 °C or incubated at 37 °C for 30 min in RPMI/H/FCS containing 0.3 mM Pq. Those incubated at 37 °C and an aliquot of those held at 0 °C were washed into DPBS/10% FCS and treated with GSH (1st). Comparison of tracks 1–3 demonstrates the presence of an intracellular pool of MHC generated by endocytosis. Aliquots of the treated cells (5×10^6) were reincubated at 37 °C for different times in the presence or absence of Pq, returned to 0 °C and then treated a second time with GSH where indicated (2nd). As a control for the stability of the label, one aliquot of cells was reincubated but not treated with GSH a second time (track 8). *b*, Bands were excised from the dried gels shown in *a*, and counted in the gamma counter. Radiolabel resistant to GSH in the α -chain of class I (○, ●) and class II (□, ■) molecules after the 2nd GSH treatment is plotted as a per cent of that resistant to the 1st GSH treatment; that is, per cent of the intracellular pool remaining. Pq was not present during the 1st GSH treatment or during subsequent washes but was then added back to some aliquots (□, ○) reincubated at 37 °C (*a*, tracks 9–12). This presumably accounts for the delayed slowing of the rate of exocytosis by Pq seen in *b*. A log-linear plot of $[(c.p.m._{(t=0)} - c.p.m._{(t=t)})/c.p.m._{(t=0)} - c.p.m._{(t=16 \text{ min})}]$ against time was linear, indicating first-order kinetics and a half life for the intracellular pools of class II and class I molecules of 2–3 min.



similar capacity to block lysosomal function in these cells. MHC glycoprotein recycling apparently continues even when endosomal acidification is compromised; interestingly, MHC-peptide exchange was also insensitive to chloroquine and monensin⁷. We suggest that some other perturbation, possibly swelling of normally acidic intracellular compartments²⁷, interferes with membrane recycling and that this is most rapidly induced by primaquine.

Recent studies have concluded that there is little traffic of cell-surface class II MHC glycoproteins through the endocytotic pathway on B lymphoblastoid cells and consequently that for presentation of antigens processed along this pathway, the relevant population is likely to consist of newly synthesized class II molecules¹². This remains an attractive hypothesis, but the experiments reported here establish that the relevant measurement is the flux through an intracellular pool rather than the pool size *per se*. If the half-time for endocytosis of the surface class II population is 33 minutes, as our data suggest, and the half-time for turnover is 36 h (ref. 13, and our unpublished data), then recycling class II traffic may exceed biosynthetic traffic by some 60-fold. The crucial question is whether the itinerary taken by recycling class II molecules includes the endosomes involved in antigen processing. This seems possible as cell-surface class II molecules were very effective as a vehicle for the delivery of a monoclonal antibody for processing and presentation to mouse immunoglobulin-specific T cells²⁸. Binding⁵, rapid turnover⁶ and even exchange⁷ of peptides occurs on living but not on lightly aldehyde-fixed cells incapable of endocytosis. The experiments reported here reveal an itinerary that might allow a single MHC class II molecule to cycle through compartments where antigen processing and peptide exchange take place and therefore to associate with different peptides during its lifetime.

The significance of class I cycling is unclear as the endoplasmic reticulum seems likely to be the normal site of association with processed antigen²⁹. Nonetheless, peptides seem to be able to bind directly to cell-surface class I^{30,31} and to isolated

class I molecules³². These sites might be generated during the recycling of labile class I/peptide complexes or monoclonal antibody/class I/peptide complexes³¹.

Finally, the combined use of cleavable cell-surface labelling reagents and Pq to slow recycling might provide a useful way of establishing whether or not a variety of surface molecules are cycling through small intracellular pools. □

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Farnesylated γ -subunit of photoreceptor G protein indispensable for GTP-binding

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TRANSDUCIN, composed of subunits $T\alpha$, $T\beta$ and $T\gamma$, is a member of a heterotrimeric G-protein family, and transduces the light signal in visual cells. We have recently found that bovine $T\beta\gamma$ can be separated into two components, $T\beta\gamma$ -1 and $T\beta\gamma$ -2, each of which has its own γ -subunit, $T\gamma$ -1 and $T\gamma$ -2, respectively¹. $T\beta\gamma$ -2 enhances the binding of GTP to $T\alpha$ in the presence of metarhodopsin II by about 30-fold compared with $T\beta\gamma$ -1 (ref. 1). Here we show that a farnesyl moiety is attached to a sulphur atom of the C-terminal cysteine of $T\gamma$ -2 (active form), a part of which is additionally methyl-esterified at the α -carboxyl group. In $T\gamma$ -1 (inactive form), however, such modifications are missing. Thus, the farnesyl moiety attached to the γ -subunit is indispensable for the GTP-binding activity of transducin. This suggests that a similar modification may occur in the γ -subunits of other heterotrimeric G proteins involved in biological signal transduction processes.

The primary structure of $T\gamma$ deduced from the complementary DNA^{2,3} (Fig. 1) has a methionine residue at the N terminus and a tetrapeptide Cys-Val-Ile-Ser at the C terminus. Both these residues are removed after translation to form mature $T\gamma$ ^{4,5}. We have reported that the apparent molecular weight of the active form of $T\gamma$ ($T\gamma$ -2) estimated from its electrophoretic mobility (M_r 6,000) differs from the inactive form ($T\gamma$ -1; M_r 8,000)¹, which agrees with the value calculated from the known sequence of mature $T\gamma$ ^{4,5}. These observations imply that mature $T\gamma$ is modified before it becomes biologically active. The C-terminal sequence of the translational product has a characteristic structure, Cys-Aaa-Aaa-Xaa, where Aaa and Xaa are, respectively, an aliphatic and any amino acid. In some proteins with this structure, the tripeptide (Aaa-Aaa-Xaa) is removed from the C terminus leaving a C-terminal cysteine which is polyisoprenylated⁶⁻⁹ and/or methyl-esterified¹⁰⁻¹³.

Amino-acid sequence analysis showed that the N terminus of $T\gamma$ -2 is a proline, consistent with mature $T\gamma$ ^{4,5}. $T\gamma$ -2 was then digested by *Staphylococcus aureus* V8 protease to produce 11 peptide fragments, which were separated by reversed-phase HPLC as shown in Fig. 2. The amino-acid sequences of all the fragments agree with a previous report⁵ (Fig. 1). Interestingly, fragments 10 and 11 (see Fig. 2) have an identical sequence (Leu-Lys-Gly-Gly) which corresponds to the C-terminal part of mature $T\gamma$ ^{4,5} (Fig. 1).

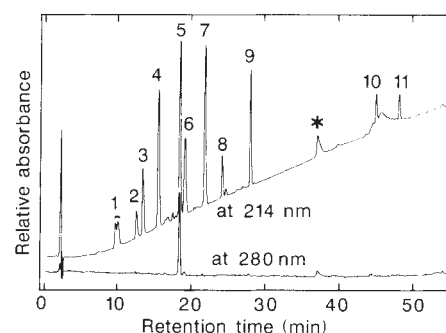


FIG. 2 Isolation of proteolytic fragments of $T\gamma$ -2. Approximately 15 μ g of $T\gamma$ -2 isolated as described¹ was digested by incubation at 37 °C for 6 h with 1 μ g of *S. aureus* V8 protease (Miles) in 30 μ l of 12.5 mM NH_4HCO_3 (pH 7.8). The digest was loaded onto a reversed-phase Cosmosil 5C₁₈-P300 column (4.6 $\times$ 150 mm; Nacalai Tesque) equipped with a HPLC system (Model LC204, Waters). Fragments were eluted with a linear gradient of 5–60% acetonitrile (1% min⁻¹) in 0.05% trifluoroacetic acid at a flow rate of 1 ml min⁻¹. The absorbances of the eluate at 214 nm and 280 nm were simultaneously monitored. A peak at a retention time of 36.9 min (marked with an asterisk) was derived from the V8 protease. A broad peak detected by the absorbance at 214 nm at a retention time of 43–47 min (which was also observed in Fig. 4) was attributed to some impurity in the solvent, because it was observed without injecting a sample. The fragments in the numbered peaks (fragments 1–11) were subjected to a gas-phase automated sequencer (Model 477A, Applied Biosystems), and identified as shown in Fig. 1. In some sequence analyses, cysteine residues of $T\gamma$ -2 reduced with dithiothreitol were pyridylethylated by 4-vinylpyridine¹⁶ before digestion. After determination of the amino-acid sequences of these fragments, amino-acid compositions of the C-terminal fragments 10 and 11 were analysed in a Hitachi L8500 amino acid analyser. Besides the three amino acids (Leu, 1.0; Lys, 1.0; Gly, 1.9, in molar ratio), 0.7 molar ratio of half-cysteine was detected in both fragments.

Fast atom bombardment (FAB) mass spectra of fragments 1–9 showed that no amino-acid residues in the peptide Pro 1–Glu 65 are modified and that the two neighbouring cysteine residues (Cys 35–Cys 36) form a disulphide bond as shown previously⁵. The spectra of fragments 10 and 11, however, displayed the parent ion peaks at m/z 681 and 695, respectively, both of which are much higher than the theoretical mass value (374) of the tetrapeptide.

On amino-acid analysis of fragments 10 and 11, a molar ratio of half-cysteine of about 0.7 was detected. These results indicate that the C-terminal amino acid of $T\gamma$ -2 is not a glycine, but a cysteine (Fig. 1) that has two different substituents (Fig. 3 inset, X_1 and X_2) in fragments 10 and 11. The accurate FAB mass spectra of fragments 10 (Fig. 3a) and 11 (Fig. 3b) showed their masses to be 681.438 and 695.453, respectively. The accurate masses of the substituents (sum of X_1 and X_2) were calculated to be 222.199 for fragment 10 and 236.214 for fragment 11 (see Fig. 3 legend). These values gave unique elemental compositions, $\text{C}_{15}\text{H}_{26}\text{O}_1$ (fragment 10) and $\text{C}_{16}\text{H}_{28}\text{O}_1$ (fragment 11), the latter having an additional CH_2 .

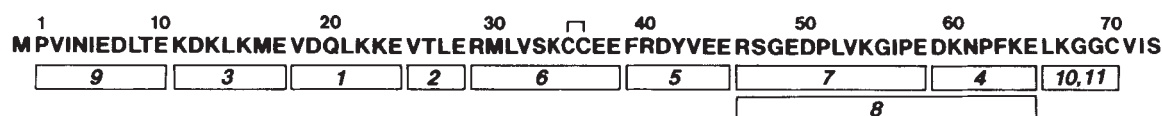
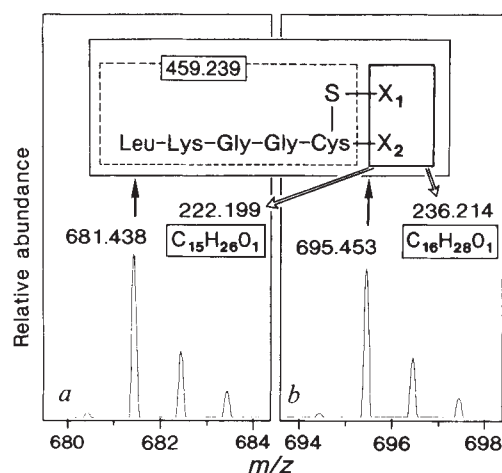


FIG. 1 Amino-acid sequence of $T\gamma$. The sequence deduced from its complementary DNA^{2,3} is given in single-letter code. Open bars indicate the fragments produced by *S. aureus* V8 protease treatment of $T\gamma$ -2 (see Fig.

2 legend), where the numbers 1–11 in italics correspond to the fragments designated in Fig. 2.

FIG. 3 Accurate FAB mass spectra of fragments 10 and 11. Lyophilized fragment 10 (a) or 11 (b) was dissolved in 50% aqueous acetonitrile, and the aliquot (~0.1 nmol peptide) was mixed with dithiothreitol/dithioerythritol (5:1, w/w) on the target. Accurate FAB mass spectra were measured by a double-focusing mass spectrometer (Jeol JMS-HX100) equipped with a FAB ion source as described¹⁷. Polyethyleneglycol 600 was used for the mass calibration. A parent ion peak with minor peaks due to the isotopic ion distribution was observed at m/z 681.438 for fragment 10 (a) or at m/z 695.453 for fragment 11 (b). The masses are averages of data obtained in five scans within an error of 0.002 mass unit. Inset, The mass of the substituents in each fragment. The accurate mass of 222.199 (fragment 10) or 236.214 (fragment 11) for the substituents (a sum of X_1 attached to the sulphur atom and X_2 to the carboxyl group of the C-terminal cysteine residue) was calculated by subtraction of the theoretical mass (459.239) of the peptide portion (enclosed with a broken line) from the observed accurate mass, 681.438 (fragment 10) or 695.453 (fragment 11), respectively. A unique elemental composition of the substituents ($X_1 + X_2$), $C_{15}H_{26}O_1$ (theoretical mass: 222.200) for fragment 10 or $C_{16}H_{28}O_1$ (theoretical mass: 236.214) for fragment 11 was obtained by using a program installed in a mass data acquisition system (Jeol JMA DA-5000).



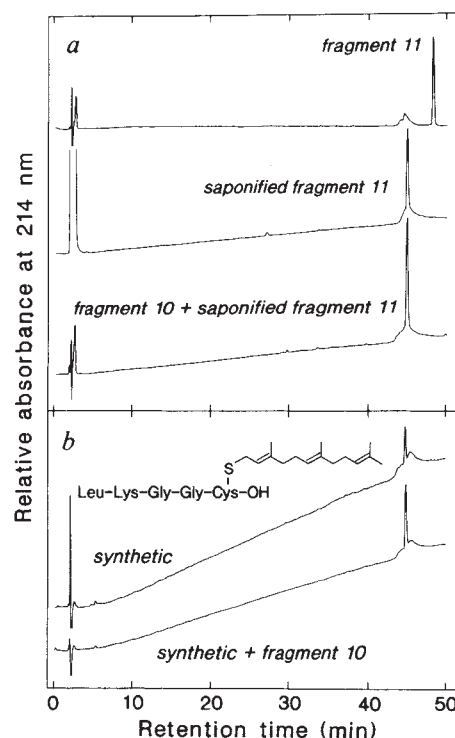
Upon alkaline treatment of fragment 11, its retention time in the reversed-phase column was shifted to that of fragment 10 (Fig. 4a). This indicates that fragment 11 could have an additional methyl group compared with fragment 10, attached through a base-labile linkage, probably as a methyl ester of the α -carboxyl group of the C-terminal cysteine. In fact, a C-terminal cysteine of a γ -subunit of bovine brain G protein was methyl-esterified *in vitro* by endogenous carboxyl methyl-transferase¹³, whose activity was also detected in bovine photo-receptor cells¹⁴. We can now identify X_1 and X_2 (Fig. 3, inset) as follows: X_1 , a farnesyl group ($C_{15}H_{25}$, in both fragments); X_2 , OH (in fragment 10) or OCH_3 (in fragment 11). The long retention time of fragments 10 and 11 in the reversed-phase column (Fig. 2) could be ascribed to the attachment of the highly lipophilic farnesyl moiety to the peptide.

To verify the structure of fragment 10, the farnesylated pentapeptide, Leu-Lys-Gly-Gly-(*S-trans,trans*-farnesyl)Cys-OH (Fig. 4b, inset), was chemically synthesized from the pentapeptide and farnesyl bromide. The peptide thus synthesized

showed the same retention time in the reversed-phase column as fragment 10 (Fig. 4b, upper trace). This was confirmed by the fact that an equimolar mixture of fragment 10 and the synthetic peptide gave a single peak in the elution profile (Fig. 4b, lower trace). In another solvent system (a linear gradient of acetonitrile in 10 mM ammonium acetate, pH 5.7), both peptides coeluted from the column. Furthermore, they both had exactly the same mass spectrum. All these results indicate that fragment 10 is identical to the synthetic *S*-farnesylated peptide. We therefore propose that Ty-2, the biologically active form, is composed of 70 amino acids (Fig. 1) and has an *S*-farnesylated cysteine at the C terminus (M_r , 8,315.7), a part of which is additionally methyl-esterified at the α -carboxyl group (M_r , 8,329.7).

The synthetic pentapeptide without the farnesyl moiety eluted from the reversed-phase column at a retention time of 5 min in the conditions described in Fig. 2 (data not shown). The absence of a peak at this position in the elution profile of the proteolytic fragments of Ty-2 (Fig. 2) indicates that all the C-terminal cysteine residues in Ty-2 are farnesylated. Parallel studies on

FIG. 4 Identification of fragments 10 and 11. The chromatographic conditions were as described in Fig. 2 legend. a, Conversion of fragment 11 to fragment 10 on saponification by alkaline treatment. Approximately 0.3 nmol of isolated fragment 11 (upper trace) was lyophilized and dissolved in 50 μ l of 0.06 M NaOH in 40% methanol, followed by incubation at 40 °C for 2 h. The pH of the solution was then adjusted to about 3 by the addition of acetic acid to quench saponification. Fragment 11 thus saponified was eluted from the column at the same retention time as fragment 10 (middle trace). Co-injection of fragment 10 and isolated saponified fragment 11 gave a single peak (lower trace). b, Identification of fragment 10 with a chemically synthesized *S*-farnesylated peptide. The synthetic peptide shown in the inset was eluted from the column at the same retention time as fragment 10 (upper trace). Isolated fragment 10 was mixed with approximately equimolar amount of the synthetic *S*-farnesylated peptide and then chromatographed similarly (lower trace). Although fragment 10 coeluted from the column with the synthetic *S-trans,trans*-farnesylated peptide, the geometry of the double bonds of the farnesyl moiety in fragment 10 has not yet been determined definitively. Synthesis of farnesylated peptide: A pentapeptide, Leu-Lys-Gly-Gly-Cys was synthesized by an automated peptide synthesizer (Model 430A, Applied Biosystems); *trans,trans*-farnesyl bromide was prepared from *trans,trans*-farnesol as described¹⁸. The farnesylated peptide was synthesized from the pentapeptide and *trans,trans*-farnesyl bromide according to the method of Kitada *et al.*¹⁹, followed by purification by reversed-phase HPLC.



T γ -1, the inactive form, indicate that it has a glycine residue at the C terminus as reported previously^{4,5}, and consequently is not farnesylated. This accounts for our observation that T γ -2 shows higher electrophoretic mobility than T γ -1¹, as farnesylation on a protein increases its mobility in gel electrophoresis⁷. Accumulation of the truncated subspecies (T γ -1) may reflect a metabolic pathway for farnesylated proteins, or alternatively suggest an unknown physiological role in photoreceptor cells. In any case, it should be emphasized that the C-terminal S-farnesyl cysteine residue of T γ is indispensable for the GTP-binding activity of transducin.

Unlike other farnesylated proteins such as nuclear lamin B⁶ and *ras* proteins⁷⁻⁹, T β γ -2 is easily extracted in soluble form. Our recent finding that α in the GDP-bound form has a much higher affinity for T β γ -2 than for T β γ -1 in the absence of membranes¹⁵ indicates that the farnesyl moiety in T γ -2 could be involved in coupling between the α - and β -subunits, rather than in membrane attachment, as proposed for *ras* proteins⁷⁻⁹.

Although the physiological role of the γ -subunit of heterotrimeric G proteins has been unclear, we now show that the farnesylated T γ is essential for interaction(s) among the three transducin subunits. This implies that a similar modification may commonly occur in all the γ -subunits of the large G-protein family.

As already described, we have detected two forms of T γ -2; carboxyl methylated and non-methylated forms. We can anticipate, however, that all T γ -2 are methylated *in vivo* and that the labile methyl group has been lost non-enzymatically during the course of isolation. This is consistent with the observation that T γ in bovine rod outer segment membranes was not labelled with [³H]S-adenosylmethionine¹⁴, probably due to the absence of the non-methylated form. Alternatively, the methylation of T γ -2 could be reversibly manipulated by an unidentified mechanism. If this is the case, the modification might regulate the light signal transduction, although to resolve this needs further study. □

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Localized requirement for *torso-like* expression in follicle cells for development of terminal anlagen of the *Drosophila* embryo

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THE *torso* (*tor*) gene, one of six identified maternal genes essential for the development of the anterior and posterior terminal structures in the *Drosophila* embryo, is likely to function as a transmembrane receptor tyrosine kinase¹. Although *tor* protein is uniformly distributed in the membrane of the egg cell and syncytial embryo², genetic^{3,4} and molecular⁵ data suggest that *tor* is locally activated at the ends of the embryo by a ligand present in the perivitelline space. Local activation of *tor* could be achieved if the ligand were expressed by a subpopulation of the follicle cells that surround the developing oocyte. Here we describe *torso-like* (*tsl*), the sixth member of the terminal gene class, and show that it is unique among these genes in that its expression is required in the somatic follicle cells rather than in the germ line. Moreover, mosaic analysis demonstrates that *tsl* expression is necessary only in subpopulations of follicle cells located at the poles of the oocyte. Thus, the spatially regulated expression of *tsl* in the follicle cell

layer may generate a localized signal that is transduced by *tor*, ultimately resulting in the formation of the terminal structures of the embryo.

Five genes have been shown to be required maternally for the development of the unsegmented terminal structures of the *Drosophila* embryo (the acron and telson): *tor*⁵, *trunk* (*trk*)⁵, *fs(1)polehole* (*fs(1)ph*)⁶, *fs(1)Nasrat* (*fs(1)N*)⁷, and *l(1)polehole* (*l(1)ph*)^{8,9}. The *tsl* gene constitutes the sixth member of this class. Embryos derived from mothers homozygous for *tsl* exhibit a phenotype virtually identical to that previously described for other genes of the terminal class (Fig. 1*b,c*). Anteriorly, the labrum is missing and the head skeleton is reduced in size, while at the posterior, abdominal segment A8 and the telson are deleted (Fig. 1*c*). As in *tor* (ref. 3), the terminal deletions in *tsl* are accompanied by an expansion of the segmented anlagen to the ends of the embryo.

The localized signal that activates *tor* is likely to be generated by other maternal genes of the terminal class. It is possible to determine which of these genes act upstream of *tor*, as would be expected for the gene encoding the *tor* ligand, by making double mutants between other terminal class genes and *tor* gain-of-function alleles. In embryos derived from mothers carrying a *tor* gain-of-function allele, terminal structures are normal, but the middle segmented region is disrupted^{3,4} (Fig. 1*d*). As these *tor* alleles are apparently constitutively active and do not require activation by the localized signal, the phenotype they produce will be unaffected by mutations in genes that are involved in generating the signal. By contrast, mutations in genes that act downstream of *tor* will prevent the expression of the gain-of-function phenotype. In combinations between *tsl*, *trk*, *fs(1)N* or *fs(1)ph* with *tor* gain-of-function alleles, the *tor* gain-of-function phenotype is epistatic over the lack-of-function terminal phenotype (Fig. 1*e,f*; see also ref. 2). Thus, all four genes act upstream of *tor* and are therefore candidates for

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encoding the *tor* ligand. The *l(1)ph* gene cannot fulfil this role as it functions downstream of *tor*^{10,11}.

We have investigated the possibility that the somatic follicle cells are involved in promoting the development of terminal structures in the embryo, and in particular, we have examined the role played by the gene *tsl*. To test whether *tsl* is required in the germ line or in the soma, five mosaic females in which the germ line was *tsl*⁻ and the somatic tissue *tsl*⁺ were constructed by pole-cell transplantations. The embryos produced by these females were wild type, indicating that *tsl* is not required in the germ line. To determine whether *tsl* must be expressed by somatic cells in the ovary itself, we carried out ovarian transplantations to create females in which the ovary was *tsl*⁻, while all other somatic tissue was *tsl*⁺ (see refs 12 and 13 for descriptions of pole cell and ovarian transplantation techniques, respectively). Progeny derived from such a female exhibited the *tsl* phenotype, indicating that *tsl*⁺ activity is required in the ovary itself, probably in the follicle cells. Three of the upstream genes, *trk*, *fs(1)N* and *fs(1)ph* are germ-line-dependent^{14,15}. Thus, *tsl* is unique among the identified terminal genes in that its expression is required in the somatic follicle cells rather than in the germ line.

The existence of polar subpopulations in the follicular epithelium has been demonstrated previously¹⁶⁻¹⁸. To test whether *tsl* expression is required in these polar follicle cells, we induced mitotic recombination to create mosaic flies with ovaries that contained clones of *tsl*⁻ follicle cells. If *tsl* expression is required only in the polar follicle cells, even a small clone of *tsl*⁻ cells, if it is located at one of the poles, will result in a *tsl* phenotype at that end of the embryo, though the other pole will be unaffected. Alternatively, if the requirement for *tsl* is not localized, both ends of the embryo should be affected equally, if at all, by *tsl*⁻ follicle-cell clones. Clone size was regulated by inducing clones at different stages of development. To determine the location of clones in the follicular epithelium, a marker mutation, *fragile chorion* (*fch*), was used. The *fch* mutation is a recessive, soma-dependent, maternal mutation that causes the chorion to be extremely thin and easily disrupted (data not shown). As the chorion is synthesized by the follicular epithelium, the location of a *fch*⁻ clone in the follicle can be deduced from the region of the affected chorion. Embryos were scored for the absence of the filzkörper, a prominent telson structure (Fig. 1a) that is deleted in embryos derived from *tsl* mothers (Fig. 1b).

When 7,180 control embryos derived from non-irradiated mothers were scored for posterior *tsl* phenotypes, none were detected. Among 10,960 experimental embryos, however, seven showed posterior *tsl* phenotypes. In these embryos, the posterior end exhibited a complete *tsl* phenotype, but normal terminal structures formed at the anterior (Fig. 2b). In addition, two of these embryos resulted from adult irradiation (see Fig. 2 legend), indicating that very small clones of *tsl*⁻ follicle cells are capable of producing a complete *tsl* phenotype at one end of the embryo. These results demonstrate that the two ends of the embryo can be affected independently, and support the idea of a localized supply of *tsl* for each pole of the embryo.

To identify the follicle cells responsible for the embryonic phenotype, the chorions of the embryos were examined for the *fch* phenotype. Six of the seven clones were associated with altered chorions. In three cases the clones were large and the chorion was disrupted, but in the remaining three the affected area was small and confined to the posterior end. The aeropyle at the posterior pole of the wild-type *Drosophila* chorion is formed by the imprints of 6-15 small central cells¹⁹ (Fig. 2c, e). These are surrounded by the imprints of 10-15 peripheral cells that are distinct both from the central and the main body imprints¹⁹. In the three clones showing localized posterior chorion defects, the main body of the chorion appears unaffected, but the central cell imprints at the posterior pole are clearly absent (Fig. 2d, f) and the peripheral cell imprints are

lacking to varying degrees. From the number of peripheral cell imprints remaining, we estimate the sizes of these clones to range from a minimum of 6 to a maximum of 30 cells (0.8-2.5% of the follicular epithelium²⁰). Thus, these chorion phenotypes were apparently caused by small clones that were restricted to the posterior pole of the follicle. The correlation of the embryonic *tsl* phenotype with a small area in the polar region of the follicle supports the hypothesis that only a polar subpopulation of follicle cells is required to express *tsl*.

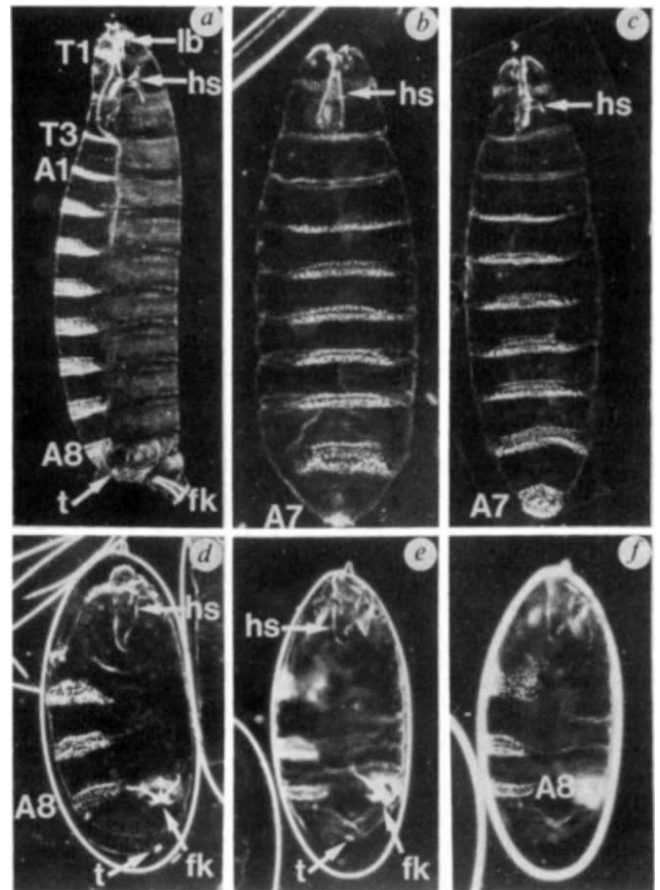
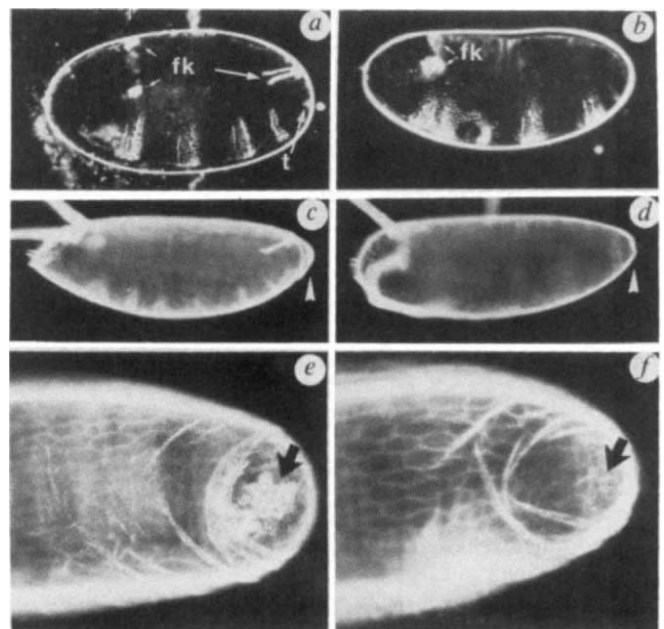


FIG. 1 Dark field photographs of cuticular preparations. Anterior, top. a, Wild-type *Drosophila* larva. Ventral, left. Thoracic (T1-T3) and abdominal (A1-A8) segments are demarcated by the ventral denticle belts. Although most of the head skeleton (hs) originates from segmented anlagen, the labrum (lb) derives from the unsegmented acron. At the posterior, the unsegmented telson includes the filzkörper (fk), anal plates and tuft (t). b, A *tsl* phenotype (maternal genotype: *tsl*¹⁴⁶/*tsl*¹⁴⁶). Ventral view. At the anterior, the labrum is lacking and the head skeleton is reduced in size. Posteriorly, A8 and the telson are deleted. c, A *tor* null phenotype (maternal genotype: *tor*^{XR1}/*tor*^{XR1}, ref. 1). Ventral view. The phenotype is essentially identical to that described for *tsl*. d-f, Ventral view. d, A *tor* gain-of-function phenotype of intermediate strength³ (maternal genotype: *tor*^{Y9}/*tor*⁺, 25 °C). Gain-of-function *tor* alleles produce a phenotype opposite to that of *tor* lack-of-function mutations. The head and telson are normal or even enlarged, but the segmented region is disrupted and segments deleted to variable degrees. e, f, Two focal planes of a double mutant between a *tor* gain-of-function allele and *tsl* (maternal genotype: *tor*^{Y9}/*tor*⁺; *tsl*⁶⁹¹/*tsl*⁶⁹¹, 25 °C). The phenotype is equivalent to that of *tor*^{Y9}/*tor*⁺ alone, indicating that *tsl* functions upstream of *tor*.

METHODS. Five ethylmethane sulphonate (EMS) induced *tsl* alleles were recovered in systematic screens for maternal-effect mutations on the third chromosome (G. Jürgens, K. V. Anderson, R. Lehmann, H. G. F. and C. N.-V., unpublished results). A sixth allele, *tsl*^{MK}, is a weak spontaneous mutation that was discovered fortuitously in a *tor*^{RL3} stock. The *tsl* gene maps meiotically to 3-71. Its cytological position is 93E, on the basis of its inclusion within *Df*(3R)^{E4} and its exclusion from *Df*(3R)^{e07} and *Df*(3R)^{eR1}.

FIG. 2 Dark field photographs of cuticular and chorion phenotypes of experimental and control embryos. Anterior, left. To facilitate the scoring of the anterior phenotype of the embryo, the mosaic analysis was done using mothers homozygous for the maternal mutation *bicoid* (*bcd*), which acts independently of *tsl* (ref. 21). In the progeny of *bcd* females, head and thorax are deleted and the acron is replaced by a duplicated telson²³. Maternal genotypes: (a) *bcd^{E1}fch⁰⁵⁵/bcd^{E1}fch⁰⁵⁵*, (b-f) *bcd^{E1}fch²⁶⁷tsl⁶⁹¹/bcd^{E1}fch⁺tsl⁺*. Embryos in b, d and f were produced by mothers irradiated as larvae (b, d), or as adults (f), and presumably derived from follicles containing *fch⁻tsl⁻* clones. Embryos in c and e are non-mosaic embryos obtained from non-irradiated females. a, The *bcd fch* phenotype. These embryos usually lack the chorion, but development of both telsons is normal. As in *bcd* embryos, the anterior filzkörper (fk) form patches rather than the filaments seen at the posterior. b, Cuticular phenotype of experimental embryo. The chorion, which has been removed in order to photograph the cuticle, was disrupted by a large *fch* clone that included the posterior pole region. Telson structures are present at the anterior, but not at the posterior pole. c, e, Chorion of control embryos. Telson and aeropyle (c, arrowhead) are present. Forming the aeropyle are the bright imprints of seven central cells (e, arrow); in some embryos the number is as high as fifteen¹⁹. d, Chorion of experimental embryo. Aeropyle is absent (arrowhead). The cuticular phenotype, which is easily detectable in the microscope, even with the chorion intact, is equivalent to that of the embryo in b. The *tsl* gene has no effect on the chorion structure; in a *fch⁺* background, embryos derived from *tsl* homozygous mothers have a normal chorion. Therefore, the absence of the aeropyle in the chorion of this embryo must be attributed to the presence of a *fch⁻tsl⁻* clone in the posterior pole region of the follicle. f, Experimental embryo in same orientation as control in e. The imprints of the central cells are absent (arrow), but the rest of the chorion appears normal. This suggests that the clone consisted of ~7–5 cells and was restricted to the posterior pole region of the follicle. The cuticular phenotype is identical to that of the embryo in b, indicating that relatively small clones are capable of producing a complete *tsl* phenotype at one end of the embryo. One of the seven embryos with a posterior *tsl* phenotype did not exhibit a chorion phenotype. This may have resulted from recombination between *fch* and *tsl*, producing a *fch⁺tsl⁻* clone. Alternatively, this clone could have consisted of cells that are required only for *tsl* expression and not for chorion production. The relationship between the *tsl*-expressing cells and those that secrete the chorion of the embryo is not known. But there is a small subset of polar follicle cells that does not synthesize chorion proteins²⁴. If these cells correspond to those required to express *tsl*, then very small clones that consist primarily or exclusively of this subpopulation would result in an embryonic *tsl* phenotype in the absence of a chorion phenotype.



METHODS. Mitotic recombination was induced by X-irradiation (1,050 R). Large clones were produced by irradiating 1st and 2nd instar larvae^{25,26}, in which follicular cell precursors are still dividing²⁰. To produce small clones, adult flies were irradiated. In adult females, recombination can be induced in follicle cells that will undergo only three to four subsequent cell divisions²⁰, resulting in a clone of ≤ 16 cells. Irradiated mothers were of the genotype *bcd^{E1}fch²⁶⁷tsl⁶⁹¹/bcd^{E1}fch⁺tsl⁺*. The genes have the following map locations: *bcd*, 3–48; *fch*, 3–55; *tsl*, 3–71. Irradiation-induced mitotic recombination preferentially occurs near the centromere²⁷ and creates cells that are homozygous for all three mutations. Irradiated females were put into egg-collection chambers with wild-type males. After adult irradiation, only eggs laid within four days were examined, to restrict the analysis to small clones. Embryos were washed gently in 0.7% NaCl, 0.02% Triton X-100, fixed with 4% paraformaldehyde in 0.1M PIPES buffer pH 6.9, 2 mM MgSO₄, 1 mM EGTA, then mounted in 50% Hoyer's/50% lactic acid.

Of 12,340 experimental embryos scored for anterior *tsl* phenotypes, only one was detected. This may have resulted from a failure to fertilize eggs with a *fch* phenotype at the anterior end. More than 90% of the eggs laid by *fch* homozygous mothers are not fertilized, probably due to anterior chorion defects.

Genetic and molecular data predict that the ligand for *tor* is present outside the egg cell and embryo and is spatially restricted to the polar regions. We provide evidence that the product of the gene *tsl* fulfils these requirements and thus may be the ligand for *tor*. Our data are also compatible, however, with the possibility that *tsl* acts further upstream, to regulate the expression of the ligand. It should be noted that our results address only the requirement for *tsl* expression, and do not provide direct evidence for its pattern of synthesis. It is possible, for instance, that *tsl* is expressed throughout the follicular epithelium and another gene product is responsible for conferring spatial specificity. The simplest interpretation of our results, however, is that the expression of *tsl* itself is the spatially regulated component of this pathway. In the anterior and posterior maternal systems, polarity is achieved through the localization of gene products in the germ-line derivatives²¹. By contrast, for the development of the terminal regions, spatial cues may be introduced into the egg cell through the differential expression of a gene product in the somatic follicle cell layer. Terminal development thus seems to be an inductive process in which pattern is imposed on the embryo according to the spatial arrangement of cells in the follicular epithelium. The communication of

information from the follicle cells to the egg is also required for the establishment of the dorsal-ventral axis in the *Drosophila* embryo²² (D. Stein, S. Roth and C. N.-V., unpublished results; T. Schüpbach, personal communication). For the terminal and dorsal-ventral systems, then, the development of polarity within the follicle cell layer may be one of the first steps in a process that ultimately determines pattern in the embryo. □

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Transcriptional activation domain of the muscle-specific gene-regulatory protein myf5

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THE human muscle determination factor myf5, like MyoD (ref. 1) and other members of the family of skeletal muscle-specific regulatory proteins^{2–7}, contains a highly conserved putative helix–loop–helix domain⁸. In MyoD this motif is required for the initiation of myogenesis in C3H mouse 10T1/2 fibroblasts⁹ and other non-muscle cells¹⁰ as well as for transcriptional activation of muscle genes. High affinity DNA binding of MyoD to regulatory DNA elements in muscle genes^{11,12} requires the formation of heterodimers with ubiquitous helix–loop–helix proteins such as E12 or E47 (refs 13, 14). To investigate the potential of myf5 as a transcription factor, we have fused the GAL4 DNA-binding domain to various parts of the myf5 protein and analysed the transactivation of a GAL4 reporter plasmid. Here we report that myf5 contains an intrinsic transcriptional activation domain which is distinct from the helix–loop–helix motif. The predominant transactivating effect is associated with the C-terminal half of the myf5 molecule. High-affinity sequence-specific DNA binding of myf5 also requires hetero-oligomeric association with the enhancer-binding protein E12 to confer muscle-specific transactivation.

The subcellular localization of myf5 in muscle cells was analysed by immunohistochemical staining with myf5-specific antiserum. In both BC3H1 myogenic cells¹⁵ and CV1 cells infected with a recombinant vaccinia virus expressing myf5, the protein was primarily localized to the nucleus with minor staining detectable in the cytoplasm (Fig. 1). This indicates that myf5, like MyoD⁹, is a nuclear protein.

The ability of myf5 to activate transcription of a muscle gene was demonstrated using the promoter region of the human embryonic myosin light chain (MLC1emb) gene fused to the chloramphenicol acetyltransferase (CAT) gene. The 465-base pair (bp) MLC1emb promoter fragment confers muscle-specific expression on the CAT construct and contains potential MyoD binding sites (E. B., T. B., B. W. and H. H. A., unpublished results). Transient expression of the reporter plasmid in 10T1/2 fibroblasts was strictly dependent on the coexpression of myf5 protein (pEMSV-myf5) (Fig. 2). This activity was stimulated by the additional expression of E12, which by itself had no effect on MLC1emb promoter activity.

To investigate whether myf5 and E12 bind directly to the MLC1emb promoter, gel mobility-shift assays were performed with both proteins prepared by cell-free translation of the corresponding complementary RNAs in rabbit reticulocyte lysates. *In vitro* translated myf5 protein alone, or together with cotranslated myf4 (myogenin⁴), did not bind to DNA *in vitro* (Fig. 3, lanes 1–2). In contrast, when cotranslated myf5 and E12 proteins

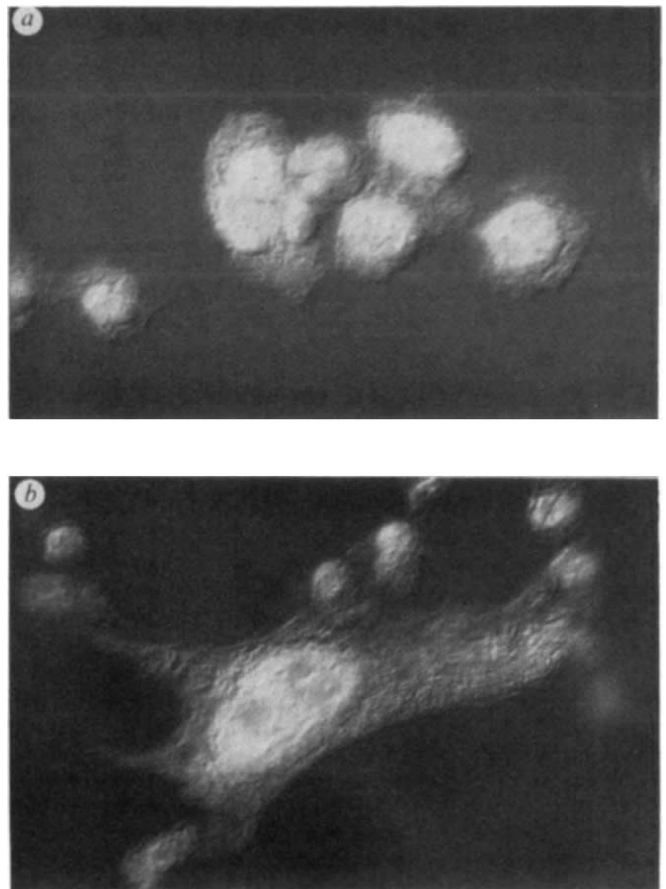


FIG. 1 Immunohistochemical localization of myf5 to the nucleus of BC3H1 cells (a) and CV1 cells infected with myf5 recombinant vaccinia virus (b). Light areas indicate myf5 antigen (negative photograph). Cells were stained with myf5 antiserum and horse radish peroxidase conjugated goat anti-rabbit IgG, and processed for peroxidase enzyme-linked immunosorbent assay (ELISA) according to the procedure suggested by the supplier of the Vecstatin ABC Kit.

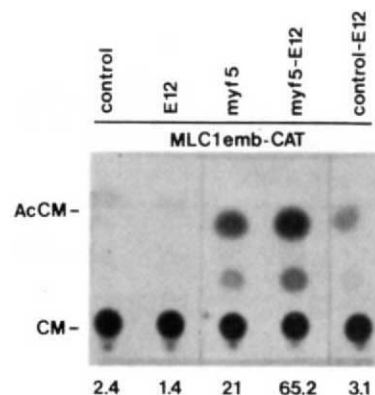
were simultaneously added to the DNA a prominent protein–DNA complex was obtained (lane 3). The addition of antiserum specific for myf5 or E12 resulted in a super-shift (lane 4) and inhibition of the complex (lane 5), respectively. This indicates that both myf5 and E12 proteins are components of the DNA-binding complex. Unlike myf5, *in vitro* translated E12 alone interacted with the muscle-specific oligonucleotide probe (lane 6). The homo-oligomeric E12 complex, however, was readily competed out by increasing concentrations of myf5, indicating that the heterodimeric or hetero-oligomeric E12–myf5 complex (higher-order associations cannot be excluded by our data) was more effective in binding to the oligonucleotide than the pure E12 complex (lanes 7–9). By analogy with MyoD it is reasonable to assume that protein–protein interactions of myf5 also involve the helix–loop–helix (HLH) structural domain^{14,16}.

To determine the transcription activating potential of myf5, independent of its ability to bind DNA, we constructed hybrid genes in which the sequence encoding the yeast GAL4 DNA-binding domain (amino acids 1–147) was fused in frame to the entire myf5 coding sequence or to various parts of it. Hybrid genes under the transcriptional control of the simian virus 40 (SV40) early promoter¹⁷ were transfected into 10T1/2 fibroblasts or HeLa cells together with the reporter plasmid 17M₂G–CAT containing the bacterial CAT gene, the β -globin TATA box, and two upstream GAL4 binding sites¹⁷. In these experiments, DNA binding is mediated by the GAL4 part of the fusion

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FIG. 2 Myf5-dependent activation of LCemb-CAT in 10T1/2 fibroblasts. C3H mouse 10T1/2 cells were cotransfected with the muscle-specific reporter construct LCemb-CAT and each of the following expression vectors pEMSV-scribe plasmid (lane 1), pEMSV-E12 (lane 2), pEMSV-myf5 (lane 3), pEMSV-myf5 plus pEMSV-E12 (lane 4), and pEMSV-scribe plus pEMSV-E12 (lane 5). Cells were incubated in DMEM medium plus 10% FCS for 48 h after transfection and CAT activity was determined. The figures indicate the percentage of chloramphenicol (CM) conversion to the acetylated derivatives (AcCM) under standard reaction conditions. The result of one representative assay is shown.

METHODS. The reporter construct LCemb-CAT contains 465 bp of the human MLC1emb gene promoter and will be described in detail elsewhere. Plasmid pEMSV-E12 contains the *EcoRI* insert of plasmid pBS ATG E12 (ref. 13) in pEMSV-scribe²¹. Plasmid pEMSV-myf5 was previously described⁸. Transient transfections were performed per 5×10^5 cells with 10 μ g of the CAT reporter construct, 1 μ g of expression vectors, 5 μ g of RSV- β -gal plasmid²² as internal control and carrier DNA to obtain 20 μ g total concentration. CAT and β -galactosidase activities were determined in 0.5–1% and 10% of the cellular extracts, respectively.



proteins, and activation of CAT activity can only be expected if myf5 contains a transcription activation domain.

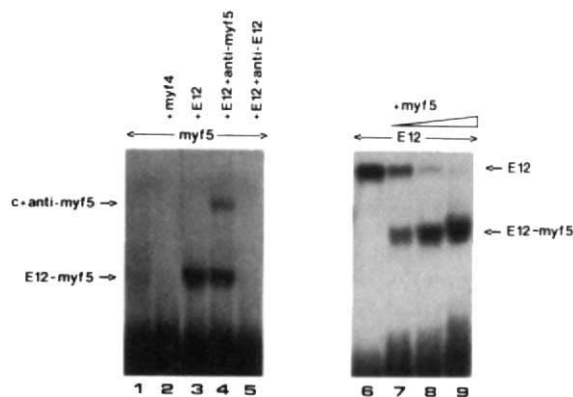
As shown in Fig. 4, the GAL4 DNA-binding domain showed no transactivation (lane 1). The complete GAL4 transcription factor (pGAL4, lane 2), and the complete myf5 protein in addition to the GAL4 binding domain (pGALmyf5, lane 3) effectively stimulated transcription of the reporter CAT construct. Similarly, the GAL4 DNA-binding domain fused to the complete E12 protein (pGAL-E12, lane 10) showed considerable transcriptional activation. In contrast, the GAL4-DBD (DBD) fusion plasmid expressing only the HLH domain of E12 (lane 11), did not activate transcription, although the protein was certainly made in the transfected cells.

The intracellular accumulation of the various fusion proteins was determined in whole-cell extracts with GAL4-specific antibodies (gift from M. Ptashne) on western blots (data not shown). Our results agree with those of Henthorn *et al.* who showed that two ubiquitous HLH proteins, E2-2 and E2-5, contain a transcription activating region outside the HLH domain¹⁸.

To dissect the regions responsible for transcriptional activation within the myf5 protein, the N-terminal so-called acidic domain (pGALmyf5 (1–46)), the conserved HLH motif including the basic region (pGALmyf5 (47–149)), and the C-terminal half (pGALmyf5 (135–255)) were analysed separately. The possible effect of the HLH domain was also investigated in conjunction with the N-terminal portion (pGALmyf5 (1–149)) or the C-terminal half (pGALmyf5 (47–255)) or in a construct lacking

the HLH motif (pGALmyf5 (Δ 46–135)). The N-terminal region (pGALmyf5 (1–46)) stimulated CAT activity only threefold (lane 4), whereas the C-terminal half downstream of the HLH domain (pGALmyf5 (135–255)) caused a tenfold increase in CAT activity (lane 6). This activation was even potentiated with a plasmid (pGALmyf5 (Δ 46–135)) lacking only the HLH motif (lane 9). The increase in activation for this construct may be the result of juxtaposing the N-terminal acidic domain and the C-terminal half. The significant stimulatory effect of the C terminus was also observed for the plasmid pGALmyf5 (47–255) (lane 8), whereas pGALmyf5 (1–149) lacking the C terminus did not activate (lane 7). The vector pGALmyf5 (47–149) expressing only the HLH domain fused to the GAL4 DNA-binding domain failed to stimulate transcription (lane 5).

Our results suggest that the myf5 protein contains a transcriptional activation domain in the C-terminal half of the molecule outside the HLH domain. Both myf5 and E12, representing tissue-specific and ubiquitous HLH proteins respectively, constitute bona fide transcription factors which do not require the putative amphipathic HLH domain for the actual stimulation of transcription. *In vitro* binding experiments presented here and elsewhere^{14,16,19} indicate, however, that sequence-specific high-affinity DNA binding of myogenic factors clearly depends on heterodimerization with E12 or E47 mediated by the HLH motifs^{14,16}. Tapscott *et al.*⁹ observed that expression of the basic and Myc similarity region (HLH motif) of MyoD was sufficient to initiate myogenesis. It seems possible therefore, that the



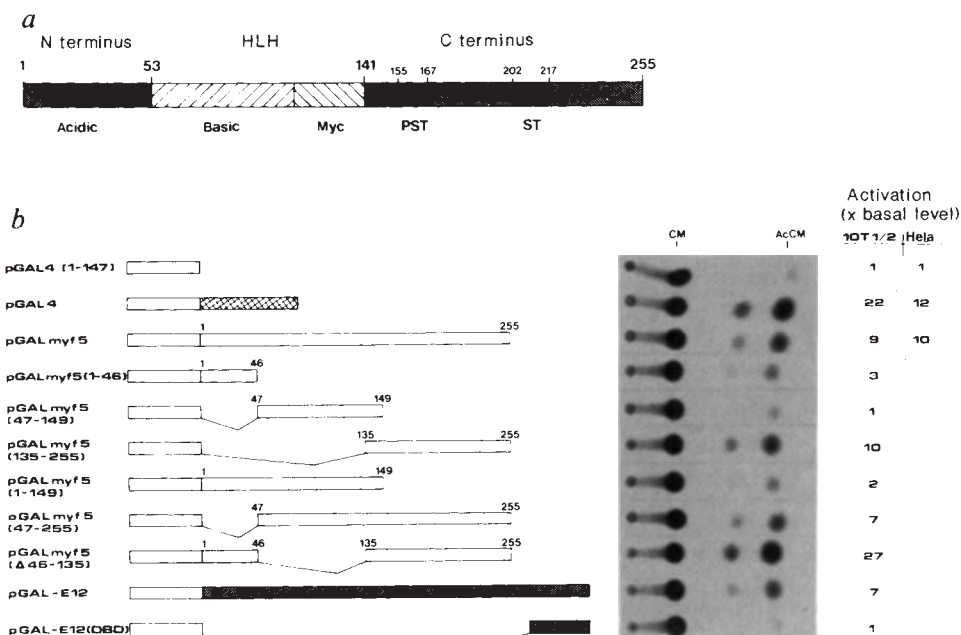
MLC1EMB: GATCAATACACAGTTGTCTGACGTGACCTGCT

FIG. 3 DNA binding of *in vitro* translated myf5-E12 protein to MLC1emb oligonucleotide encompassing the potential MyoD-binding consensus sequence (underlined) in the human MLC1emb gene promoter. Equal concentrations of *in vitro* translated myf5 protein (lanes 1–5) were incubated with radiolabelled MLC1emb oligonucleotide and either of the following *in vitro* translated proteins and antisera: myf4 (lane 2), E12 (lane 3), E12 plus anti-myf5 IgG (lane 4), and E12 plus anti-E12 IgG (lane 5). The positions of myf5-E12 and E12 complexes are indicated. Anti-myf5 IgG was raised in rabbits and leads to a supershift of the E12-myf5 complex. Anti-E12 serum prevents complex formation. In lanes 6–9 equal concentrations of *in vitro* translated E12 were mixed with increasing amounts of myf5 (in times amount of E12: lane 7, 0.3 $\times$; lane 8, 0.6 $\times$; lane 9, 1 $\times$). The homo-oligomeric E12 complex is larger than the hetero-oligomeric E12-myf5 complex, as indicated by arrows.

METHODS. Complementary RNAs for myf5 and E12, generated with T3 or T7 RNA polymerase from linearized plasmid templates pEMSV-myf5 and pBS-ATG-E12 respectively, were translated in pretreated rabbit reticulocyte lysate (Promega) according to standard procedures. The relative yields of proteins myf5 and E12 were estimated by the incorporation of [³⁵S] methionine into each protein taking into account the total number of methionine residues per molecule. Incubation conditions and PAGE have been described previously²³.

FIG. 4 Identification and tentative mapping of the transactivator region in the myf5 protein. **a**, Schematic representation of structural domains in the myf5 protein, indicating the HLH segment and the N- and C-terminal regions (shaded). PST, block of conserved proline, serine and threonine residues; ST, conserved serine- and threonine-rich sequence. The N-terminal sequence contains ~30% acidic amino acids, partly in clusters, and is therefore referred to as the acidic domain. The putative HLH structure is presented as basic and Myc-homology domains in reference to the originally suggested structure for MyoD1 (refs 1, 9). The basic domain encompasses the upstream cluster of basic amino acids, helix 1, and the putative loop segment. The Myc domain is now referred to as helix 2 (ref. 13). The figures indicate amino-acid residues in myf5. **b**, Transcriptional activation of the reporter plasmid 17M₂G-CAT (ref. 17) containing two GAL4 binding sites, tested with several chimaeric plasmids expressing all or parts of myf5, and the entire or the HLH domain of E12. The various hybrid expression plasmids are shown schematically. The GAL4 DNA-binding domain was generally supplied by the coding information for the amino acids 1–147. Numbers refer to the myf5 amino acids expressed by the various constructs. A representative CAT assay of transiently transfected 10T1/2 cells is shown. The numbers represent means of five independent transfections for 10T1/2 cells and three for HeLa cells. Values for activation are expressed as multiples of CAT activity with reference to the basal level (of pGAL (1–147)) set as 1.

METHODS. Chimaeric pGALmyf plasmids were constructed using appropriate *Bam*HI linkers to maintain the open reading frame in the expression vector



pG4MpolyII (ref. 17). Plasmid pGAL-E12 was also constructed by cloning the 1.2-kilobase E12 cDNA by *Bam*HI linkers in pG4MpolyII. The E12 HLH domain was taken as *Sma*I-*Eco*RI 280-bp fragment from the 3' end of pBs-ATG-E12 (ref. 13). Transient CAT assays were performed in 10T1/2 or HeLa cells by transfection by 1 μg of the various expression vectors along with 1 μg 17M₂G-CAT reporter plasmid of 4 μg of RSV-β-gal plasmid²² as internal standard. CAT activity was determined 3 days after transfections and values were normalized to β-galactosidase activities to calibrate for different transfection efficiencies.

putative intracellular protein complex composed of the MyoD HLH domain and complete E12 sufficiently stimulates transcription to start the positive autoregulation of the muscle determination genes^{4,20}.

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Cell cycle-dependent regulation of histone precursor mRNA processing by modulation of U7 snRNA accessibility

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HISTONE gene expression is regulated both during and after transcription¹, with the turnover of histone messenger RNA and the regulation of its 3' processing being the principal factors that determine the size of the histone mRNA pool during the cell cycle².

The mature ends of the replication-dependent histone mRNAs are generated during 3' processing by endonucleolytic cleavage of a large primary transcript³. This reaction depends on a highly conserved stem-loop structure also included in the mature RNA, and a purine-rich sequence lying downstream within the spacer transcript^{4,5}. Three factors involved in this reaction which act *in trans* have been identified: the U7 small nuclear ribonucleoprotein particle (snRNP) (refs 6–9), the heat-labile factor¹⁰, and the hairpin-binding factor^{7,11}. Here we show how U7 snRNP participates in the regulation of 3' processing: the 5' sequences of the U7 snRNA that hybridize with the downstream spacer motif during 3' processing¹² are occluded in the G0 stage of the cell cycle but are exposed and free to interact with histone pre-mRNA during S phase, when histone mRNA synthesis is at its peak¹³.

Nuclear extracts of exponentially growing cells, when heated to 50 °C for 15 min, lose their capacity to process the 3' end of

histone pre-mRNAs *in vitro*. These extracts can be activated, however, by addition of untreated nuclear extracts or their subfractions¹⁰: for example, activity is restored when a heated extract, containing U7 snRNPs, is complemented by an extract containing the heat-labile factor but free of U7 snRNPs (Fig. 1, lane 10). C3H10T1/2 cells can be arrested in G0 when starved of serum (0.5% serum; ref. 14, and L. Philipson, personal communication) and can be chased into G1 and S phase by re-addition of serum¹⁴. FACSCAN analysis shows that >90% of the cells are in G0 (or G1) after serum starvation (data not shown) and that S phase, as assayed by [³H]thymidine incorporation, is re-established between 14 and 18 h after feeding the cells with 20% serum (Fig. 2a). We prepared nuclear extracts from cell cultures in G0 and from cultures released from G0 arrest for increasing time periods and tested their capacity to complement heated extracts from exponentially growing C3H10T1/2 cells. We found that nuclear extracts from cells arrested in G0 were not effective in complementing extracts: 3' processing remained low at best (Fig. 2b, lanes 5 and 6), and so we judged these G0 nuclear extracts to be virtually depleted of heat-labile factor. Complementation activity increased noticeably after 14 h, that is, immediately before the re-establishment of DNA synthesis; by 18 and 24 h it was comparable with that of a nuclear extract from exponentially growing cells at 18 and

24 h. These results are consistent with the down-regulation of heat-labile factor activity observed in cells arrested in G1¹⁵.

We treated nuclear extracts from the same series of cell cultures at G0, or released from G0, with micrococcal nuclease and measured the accessibility of their U7 snRNA sequences to enzyme digestion by mapping the U7 snRNA sequence with radioactive antisense U7 RNA (see ref. 16 for details). This procedure preferentially removes 21 of the 5' terminal nucleotides of the U7 snRNA in extracts from exponentially growing

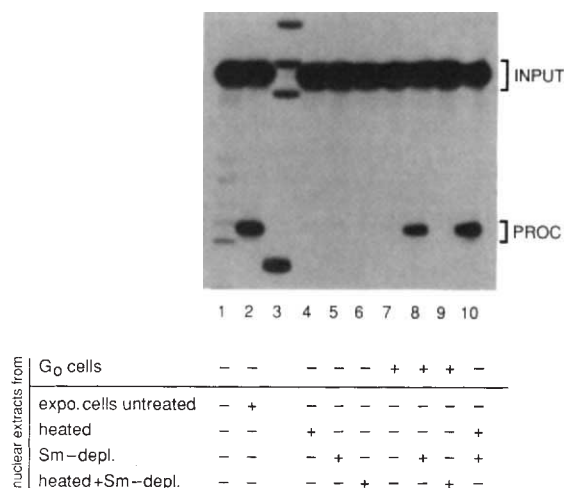


FIG. 1 *In vitro* 3' processing showing complementation of snRNP (Sm)-depleted nuclear extracts with nuclear extracts from cells arrested in G0. Lane 1, Histone pre-mRNA input; lane 2, control processing reaction of exponentially growing cells; lane 3, *Hpa*II digest of pBR322; lane 4, processing reaction with heat-inactivated nuclear extract from exponentially growing cells (expo); lane 5, processing reaction with Sm-depleted nuclear extract of exponentially growing cells; lane 6, heat-inactivated and Sm-depleted nuclear extract from exponentially growing cells; lane 7, nuclear extract from G0-arrested cells; lane 8, complementation between Sm-depleted nuclear extract of exponentially growing cells and extract from G0 arrested cells; lane 9, complementation attempt between Sm-depleted and heat-treated nuclear extract of exponentially growing cells with nuclear extract from G0 arrested cells; lane 10, complementation between heat-inactivated nuclear extract from exponentially growing cells and Sm-depleted nuclear extract from exponentially growing cells. Note that nuclear extracts from G0-arrested cells (lane 7) do not support 3' processing, but such extracts can apparently provide active U7 snRNPs because they can support 3' processing together with Sm-depleted, non-heated nuclear extracts from exponentially growing cells (lane 8). If such complementing Sm-depleted extracts from exponentially growing cells are heat-inactivated at 50 °C, there is no complementation (lane 9). Histone pre-mRNA is designated 'input' and the processed RNA as 'proc'.

METHODS. snRNP depletion of nuclear extracts was as described¹⁰, using anti-Sm serum K (gift from E. de Robertis). Nuclear extract (25 µl) was depleted using 60 µl serum. Capped histone precursor RNA was produced with T7 polymerase off the mouse histone H4 gene¹¹ and contained 21 nucleotides upstream and 33 nucleotides downstream of the 16-nucleotide conserved histone mRNA palindrome.

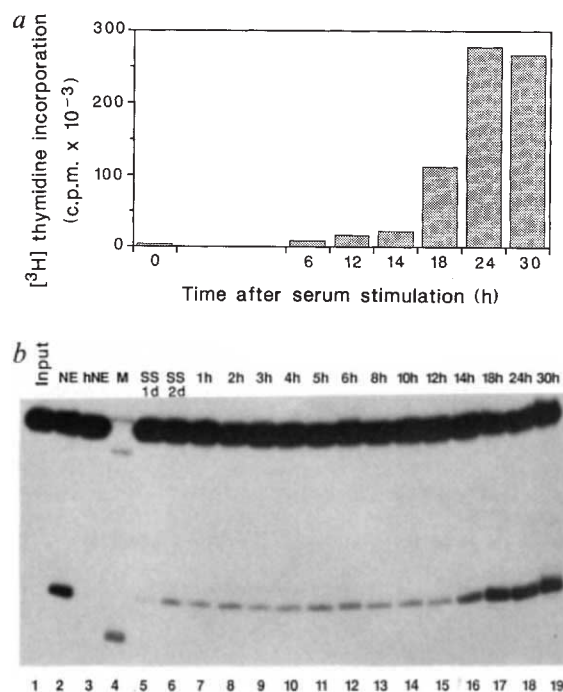
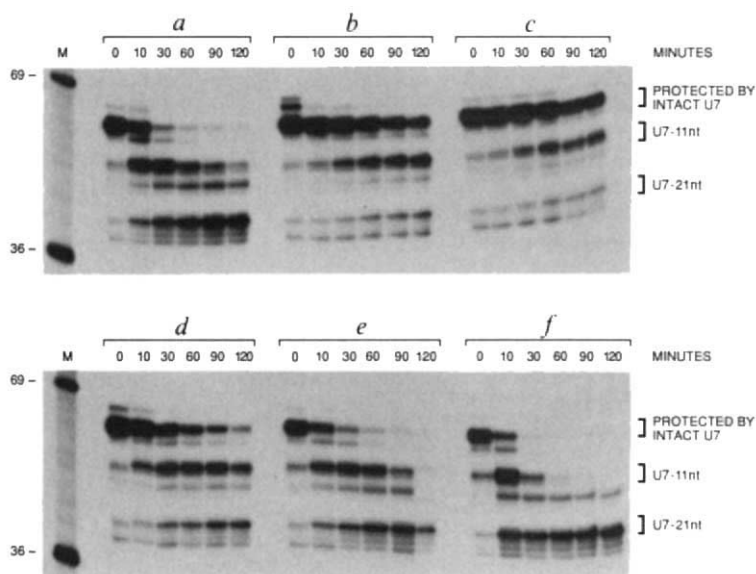


FIG. 2 **a**, [³H]thymidine incorporation (in c.p.m. per 5 × 10⁵ cells) after serum-stimulation of G0-arrested C3H10T1/2 cells. **b**, Time course of the return of heat-labile factor activity after serum stimulation of cells arrested in G0. Mouse C3H10T1/2 cells were cultured in Dulbecco's modified Eagle's medium with 10% FCS. For serum starvation, the cells were washed in PBS and then transferred to 0.5% FCS when the cultures were 50% confluent. Cells were left in the same medium for 2 days. DNA synthesis was induced by the addition of fresh medium containing 20% FCS. At each time point indicated, cells were pulsed with [methyl-³H]thymidine (8 µCi ml⁻¹) for 60 min, washed in PBS, collected, precipitated with trichloroacetic acid (10% final concentration) and the c.p.m. determined. Another set of cells were collected at different times after FCS stimulation for the preparation of nuclear extracts. These were tested for the presence of the heat-labile factor by standard complementation experiments. Lane 1, Histone pre-mRNA input; lane 2, control processing reaction with nuclear extract (NE) from rapidly growing cells; lane 3, heat-treated nuclear extracts (hNE); 4, calibration *Hpa*II-digest of pBR322(M); lanes 5 and 6, cells serum-starved (SS) for 24 h and 48 h respectively; lanes 7–19, heat-labile factor activity of nuclear extracts prepared after serum stimulation for the indicated times and mixed with heat-treated nuclear extract (lane 3).

METHODS. Preparation of miniature nuclear extracts has been described¹⁸. Cells were collected using a rubber policeman and washed in 20 volumes of PBS, resuspended in one packed cell volume of buffer A (10 mM HEPES/K⁺, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT) and allowed to swell on ice for 15 min. Cells were disrupted by pushing them rapidly 5 times through a 26-gauge needle. After centrifugation, the crude nuclear pellet was resuspended in two thirds of the packed cell volume in buffer C (20 mM HEPES/K⁺, pH 7.9, 25% glycerol, 0.6 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF), followed by incubation on ice with agitation for 30 min. The nuclear debris was pelleted by centrifugation and the nuclear extract was dialysed against buffer D (20 mM HEPES/K⁺, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF). We used the *in vitro* processing reaction of Gick *et al.*³. For heat-inactivation, nuclear extract from exponentially growing cells was preincubated at 50 °C for 15 min, which resulted in a loss of 3'-processing activity. Complementation (lanes 7–19) with fractions containing the heat-labile factor restores 3'-processing activity.

FIG. 3 The return of the susceptibility of the 5' end of U7 snRNA to digestion by micrococcal nuclease after serum stimulation of G0-arrested cells. RNase protection mapping of nuclear extracts from C2H10T1/2 cells treated with micrococcal nuclease (Pharmacia): *a*, exponentially growing cells; *b*, G0-arrested cells; *c-f*, G0-arrested cells stimulated with 20% FCS for 10 h (*c*), 14 h (*d*), 18 h (*e*), and 30 h (*f*). Previous experiments have shown that truncation of sequences occurs at the 5' end of the U7 snRNA^{16,17}. M, Two fragments (36 and 69 nucleotides) from a *Hpa*II digest of pBR322; U7-11 nt, resection of the U7 snRNA 5' end by 11 nucleotides; U7-21 nt, resection of the U7 snRNA 5' end by 21 nucleotides.

METHODS. Nuclear extracts isolated at different times after treatment of the cells with 20% FCS were incubated at 30 °C with micrococcal nuclease (8 U per 60 μ l nuclear extract). At the times indicated, aliquots were removed and the micrococcal nuclease inactivated with 1 μ l of 80 mM EGTA. Fractions were then treated with proteinase K (1 mg ml⁻¹) in the presence of 1.0% SDS, 300 mM NaCl, 20 mM EDTA, 20 mM Tris-HCl, pH 8.0, at 37 °C for 30 min, extracted twice with phenol/chloroform and precipitated with ethanol in the presence of *Escherichia coli* transfer RNA as a carrier. The resulting RNA was subjected to RNase protection mapping¹⁶ with a ³²P-labelled antisense U7 snRNA probe and resolved by electrophoresis through a 12.5%-8.3 M urea in TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA). The antisense RNA was prepared by transcription of a



linearized DNA template with T7 polymerase^{16,17}.

cell cultures^{16,17} and concomitantly inactivates 3' processing¹⁶.

The U7 snRNA from G0 cells remained intact and largely resistant to digestion by micrococcal nuclease even over extended periods of time (Fig. 3*b, c*). This was not a result of inactivation of the enzyme, because when we added labelled RNA probes to the extracts, they were digested to oligonucleotides within 5 min (data not shown). Therefore, we conclude that in G0-arrested cells, the U7 snRNA in U7 snRNP is largely protected from nuclease attack, either as a result of being bound to protein or RNA, or because of an alteration in its secondary structure. As cells enter S phase, the U7 snRNA becomes increasingly susceptible to micrococcal nuclease, which is first noticeable at 14 h (Fig. 3*d*). We have thus demonstrated that the sensitivity to micrococcal nuclease of the 5' terminal sequences in U7 snRNA roughly parallels the fluctuation of the heat-labile factor during the cell cycle.

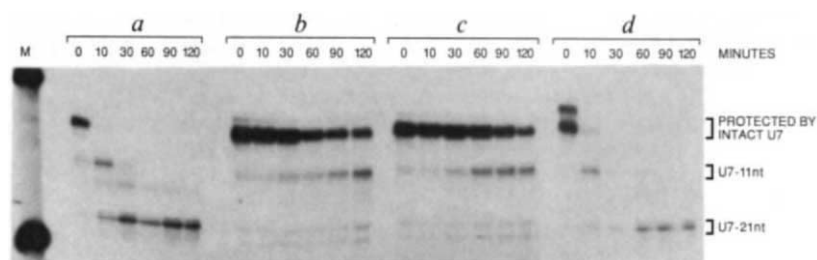
The finding that the availability of the 5' terminal sequences of U7 snRNA is regulated during the cell cycle is interesting because of the part that this snRNA sequence is known to play in the 3' processing reaction. We have shown previously¹² that base alterations in the histone pre-mRNA downstream conserved spacer sequence abolish 3' processing of the histone pre-mRNA, whereas compensatory base changes introduced into the 5' terminal sequences of the U7 RNA re-establish 3' processing. This is the best evidence so far that the 5' terminal sequences of the U7 snRNA contact, through Watson-Crick base-pairing, the downstream processing signal in the spacer of histone pre-mRNA¹². Here we show that during S phase, when histone mRNA synthesis is at its peak², the same 5' terminal sequences

are freely accessible, but that these sequences somehow become occluded in G0 and during the time between G0 and S phase, presumably G1, when there is limited, if any, processing of histone pre-mRNA (Fig. 1, lane 7) because of the deficiency of heat-labile factor (Fig. 2*b*, lanes 5 and 6).

If G0 cells contain U7 snRNP in an inactive occluded form, how is it that G0 cell extracts can support 3' processing of histone pre-mRNA when complemented with unheated extracts that have been depleted of U7 snRNP (Fig. 1, lane 8)? The simplest explanation is that complementing extracts modify the G0-type U7 snRNP *in vitro* so that 3' processing can take place. We therefore investigated the status of the terminal 5' sequences of the U7 snRNA in non-complemented G0 extracts, and in G0 extracts after complementation with heat-labile factor. As expected, the G0 extract contained U7 snRNP with largely occluded 5' terminal RNA sequences (Fig. 4*b*), whereas after addition of complementary extracts containing heat-labile factor but no U7 snRNPs, the 5' ends of the U7 snRNA became accessible to micrococcal nuclease (Fig. 4*d*), indicating that the U7 snRNP was now in the activated form.

Three findings suggest a possible link between the heat-labile factor and the accessibility of the 5' terminal sequence of the U7 snRNA. First, the appearance of the heat-labile factor and the free 5' end of U7 RNA is simultaneous in cells released from G0. Both seem to be late events in the signal cascade that drives cells from G0 into the cell cycle and proliferation after stimulation by serum. Second, addition of extracts containing heat-labile factor (Fig. 4*d*), or of extensively purified heat-labile factor (data not shown), to G0-type U7 snRNP results in the

FIG. 4 The 5' end of U7 snRNA in G0-arrested cells becomes accessible after addition of a snRNP-depleted nuclear extract. RNase protection mapping of a micrococcal nuclease digest of *a*, exponentially growing C3H10T1/2 cells and *b*, G0-arrested cells. *c*, Micrococcal nuclease assay of U7 snRNP after combining G0 nuclear extract with heat-treated and Sm-depleted nuclear extract from exponentially growing cells. Extracts were assayed immediately after mixing. Nuclear extract from G0-arrested cells was complemented with Sm-depleted nuclear extract from exponentially growing cells and immediately digested with micrococcal nuclease; this was followed by RNase protection mapping (*d*). Abbreviations M, U7-11 nt and U7-21 nt as in Fig. 3 legend.



viations M, U7-11 nt and U7-21 nt as in Fig. 3 legend.

appearance of the activated form of U7 snRNP. Third, heat-inactivated and snRNP-depleted extracts do not complement G0 extracts with respect to 3' processing (Fig. 1, lane 9) and do not cause the appearance of a free 5' end when incubated with extracts containing G0-type U7 snRNPs (Fig. 4c). At its simplest, the heat-labile factor could be an activator of U7 snRNP; however, direct proof of this must await purification of the heat-labile factor to homogeneity and the reconstruction of U7 snRNP *in vitro* from purified components. □

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Negative regulation of human *c-fos* expression by the retinoblastoma gene product

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INACTIVATION of the retinoblastoma susceptibility gene (*RB-1*) has been associated with the aetiology of many types of human cancers, leading to the classification of *RB-1* as an anti-oncogene or tumour suppressor gene^{1–13}. Given that the protein product of *RB-1* (Rb) has a nuclear localization and DNA-binding activity *in vitro*, it is possible that Rb regulates transcription of certain genes. The promoter of the *c-fos* gene might be a target for regulation by Rb, because both *v-fos* and *RB-1* are associated with the induction of osteosarcomas in mice and humans, respectively^{14,15}. Also, *fos* expression is thought to be required for quiescent cells to enter the cell cycle, making the *fos* promoter an attractive target for suppressors of cell growth^{16–18}. Here we report that Rb can repress *c-fos* expression and AP-1 transcriptional activity in both serum-induced and cycling 3T3 cells. We have mapped a *cis*-acting element in the human *c-fos* promoter that can confer repression by Rb to a heterologous promoter. We have termed the *cis*-acting sequence regulated by Rb the retinoblastoma control element.

In this study we used a human *c-fos* promoter containing sequences between –356 and +109 (ref. 19), relative to the start site of transcription, positioned directly upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene (FOS-CAT; Fig. 1). Because the product of the *fos* gene complexes with a member of the Jun family of transcription factors to give high-affinity binding to an AP-1-binding site^{20,21}, we also examined the effect

of Rb on AP-1 transcriptional activity. For a reporter of AP-1 transcriptional activity, five copies of a synthetic AP-1-binding site were positioned upstream from a truncated *fos* promoter (Δ 56CAT) containing only a TATA element (AP-1CAT; Fig. 1).

NIH/3T3 cells were transiently transfected with the reporter plasmids in conjunction with vector plasmid DNA (pJ3 Ω) or a plasmid carrying the mouse *RB* complementary DNA²² (pmRB; Fig. 1). The transfected cells were maintained in low-concentration serum for 48 h and then treated with high-concentration serum for 90 min to determine if Rb modulated *fos* expression at the point of the cell cycle (G0–G1) where Fos protein is thought to stimulate cell growth. The transcription of *fos* and AP-1 activity are low in quiescent cells, but are rapidly induced by serum. Transient expression of mouse *RB* significantly reduced stimulation of the *fos* promoter by serum (Fig. 2a, lanes 1 and 2). Concomitant with a reduction in *c-fos* promoter activity, transient expression of plasmid pmRB also reduced the serum stimulation of endogenous AP-1 transcriptional activity (lanes 3 and 4).

To determine if RB can affect the steady-state level of AP-1 transcriptional activity in cycling cells, the AP-1CAT reporter construct was cotransfected with pmRB or a plasmid expressing human Rb (phuRB). In contrast to the previous experiments, the transfected 3T3 cells were maintained in high serum (10% calf serum). Transfection of AP-1CAT with the mouse or human RB cDNA constructs resulted in a significant reduction of CAT activity (Fig. 2b, lanes 2 and 3) relative to the vector control (lane 1). Averaging the results from five independent experiments, there was a 5- and 2.5-fold reduction of AP-1 activity by

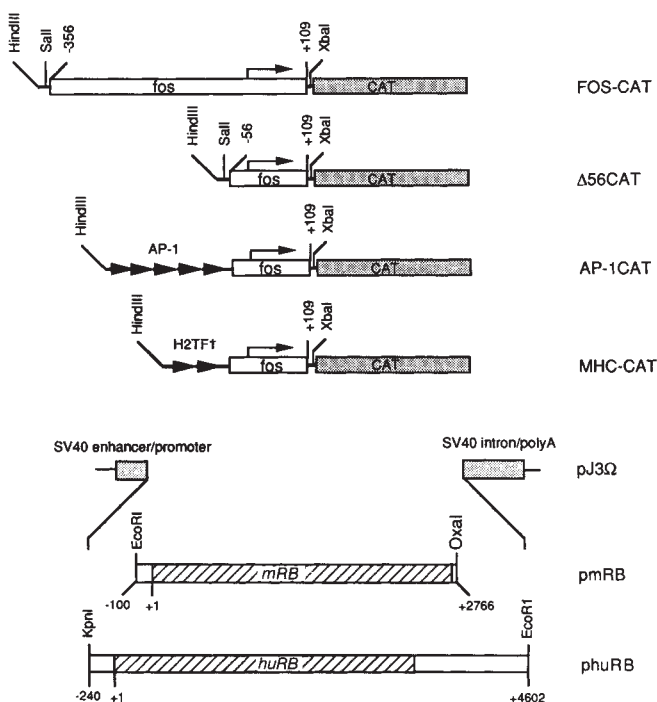


FIG. 1 Reporter and transregulator constructs. The *fos* promoter construct, FOS-CAT, contains human *c-fos* sequences between –356 and +109 positioned directly upstream from a CAT gene linked to SV40 splice and poly(A) signals. Five copies of a synthetic AP-1-binding site (GTGACTCAGCG) were inserted directly upstream from the truncated *fos* promoter at the *Sal*I site in Δ 56CAT to give AP-1CAT. MHC-CAT contains two copies of the H2TF1-binding site from the MHC promoter²³. The mouse *RB* expression vector pmRB contains an *Eco*RI (position –100) to *Oxa*N1 (position +2,766) mouse cDNA fragment cloned into the *Eco*RI and *Cla*I sites of pJ3 Ω (originally termed pJ3 Ω 115R0X; ref. 22). The human *RB* expression vector, phuRB, contains a *Kpn*I (position –240) to *Eco*RI (position 4602) human cDNA fragment cloned into the *Sma*I and *Eco*RI sites of pJ3 Ω (originally termed pJ3 Ω HRbC).

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mouse and human Rb, respectively. There was also a dose-responsive reduction of AP-1 activity by both mouse and human Rb in CV-1 cells (data not shown).

To determine whether Rb-mediated repression was specific to the *c-fos* promoter or due to global repression of cellular transcription, a $\Delta 56$ CAT construct containing two copies of a H2TF1 binding site from the human major histocompatibility complex (MHC) promoter (MHC-CAT; ref. 23) was similarly tested. No effect of Rb cotransfection on H2TF1-dependent expression was observed (Fig. 2a, lanes 5 and 6; Fig. 2b lanes 4, 5, and 6) even when the amount of protein extract used was adjusted to give a CAT signal within the linear range (data not shown). Moreover, when a RSV- β -gal expression plasmid was included as an internal control for transfection efficiency in the cotransfection assay, there was significant repression by Rb of *fos* expression in serum-induced cells (Fig. 2c) and AP-1 activity in cycling cells (Fig. 2d). On the basis of the above observations, we conclude that Rb can regulate the level of *c-fos* transcription and presume that this regulation results in a reduction in the level of AP-1 activity.

To demonstrate that a functional Rb protein is required for repression, we constructed several mouse Rb deletion mutants for analysis in our cotransfection assay. The mutations were introduced into a region of the mouse Rb cDNA thought to be of functional importance. Several human tumour cell lines carry

Rb point mutations that lead to the production of aberrant Rb proteins^{2,4}. These aberrant proteins have lost 35 or 38 amino acids encoded by exons 21 or 22, respectively, of the human Rb gene. Although these mutant proteins retain affinity for DNA, they are defective in their ability to associate with viral oncoproteins^{2,4,24-26}. Therefore, we generated three small in-frame deletions producing internally deleted proteins, and a deletion producing a truncated protein, in the mouse Rb cDNA by *Bal31* digestion beginning at restriction sites within exons 19, 20 or 21 of the mouse cDNA. Deletion DS2-3 removed two

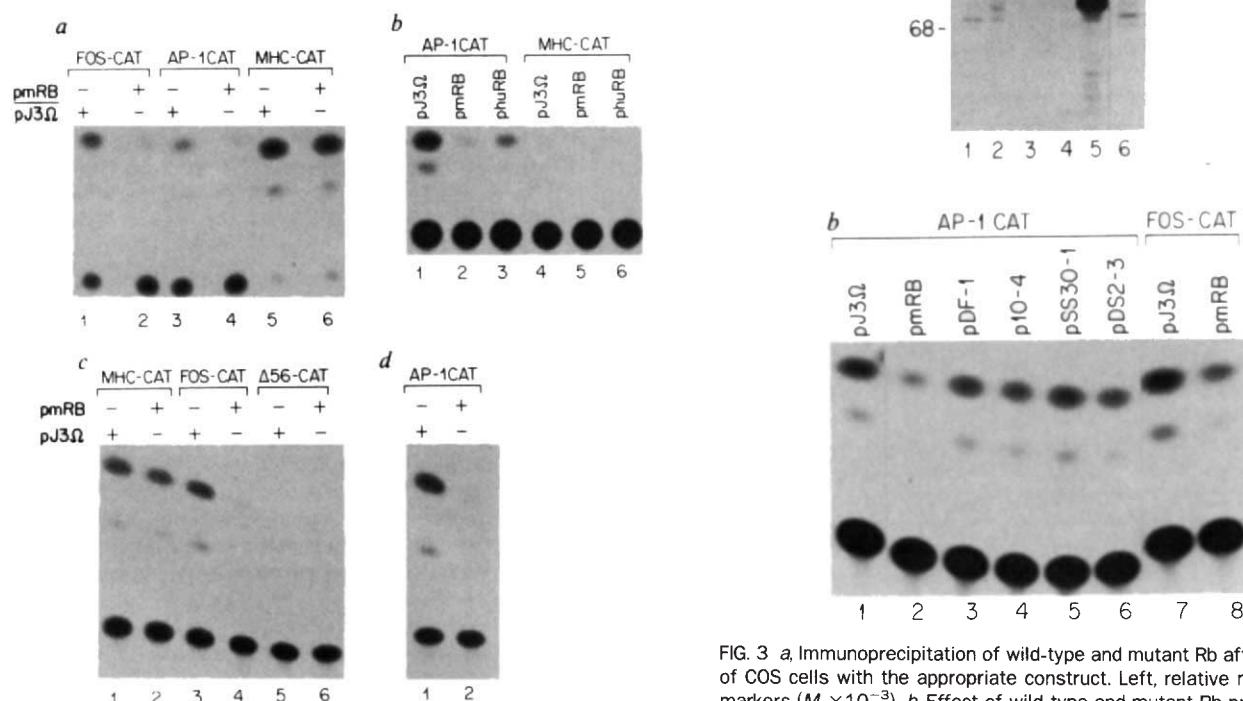


FIG. 2 Effect of mouse and human Rb on human *c-fos* expression and AP-1 activity in serum-stimulated quiescent cells. Reporter construct 1 μ g was cotransfected with 5 μ g Rb plasmid or the control vector, pJ3 Ω , using a DEAE-dextran transfection procedure. The DNA DEAE-Dextran mixture (5 ml; 50 μ g DEAE-Dextran per ml of DME) was added to a subconfluent plate of NIH/3T3 cells for 3 h in the presence of 100 μ M chloroquine followed by a 10% dimethyl sulphoxide shock for 1 min. c, d, A plasmid expressing the β -galactosidase gene from the Rous sarcoma virus LTR (RSV- β gal) (1 μ g) was included as an internal control for transfection efficiency. The level of CAT activity was determined 48 h after transfection using equivalent levels of protein extract. CAT activity was quantified by determining the percentage of acetylated 14 C-labelled chloramphenicol. Both the acetylated and non-acetylated forms of 14 C-labelled chloramphenicol were removed from the thin-layer chromatography plate and analysed using a scintillation counter to determine CAT activity. c, d, Extracts were normalized against the level of β -gal expression. a, c, Cells were placed in 0.5% calf serum immediately after transfection, then stimulated with 20% FCS for 90 min at 48 h after transfection. b, d, Cells were maintained in 10% calf serum after transfection.

FIG. 3 a, Immunoprecipitation of wild-type and mutant Rb after transfection of COS cells with the appropriate construct. Left, relative molecular mass markers ($M_r \times 10^{-3}$). b, Effect of wild-type and mutant Rb proteins on AP-1 activity in 3T3 cells. Four deletions were introduced into exons 19, 20 and 21 of the mRb cDNA at three different restriction sites using *Bal31*. The DF-1 deletion results in a truncation at the *DraIII* site at position +1970, thus removing the C-terminal 264 amino acids. The *DraIII* site is 34 nucleotides into exon 20 of the mRb cDNA, within the putative leucine-zipper sequence LNTLCAR-LLSDHPE-LEHIIWJL (single-letter amino-acid code). DS2-3 has six nucleotides deleted at the *DraIII* site at position +1,970 removing a leucine, cysteine and alanine residues and inserting a proline residue (LNTLCARL > LNTPLRL). SS30-1 was generated by a deletion at the *SacI* site at position +2,055, resulting in the loss of 37 amino acids at the 3' end of exon 20 and the beginning of exon 21, including half the putative leucine-zipper. The 10-4 mutation was created by deletion at the *BstEII* site (position +1,770) removing 14 amino acids from the 3' end of exon 18 and the 5' end of exon 19. Immunoprecipitation of the different Rb variants after transfection were performed as described⁴. Immunoprecipitation of T antigen from nontransfected COS cells was a positive control. In the cotransfection assay shown in b, a RSV- β -gal expression plasmid was included as an internal control for transfection efficiencies. The level of β -gal expression did not vary more than 20% for the different cotransfections.

amino acids within the putative leucine-zipper region of mouse *RB* and replaced an alanine residue with a proline residue. Deletion SS30-1 removed 37 amino acids including half of a putative leucine-zipper region^{27,22}. Deletion 10-4 removed 14 amino acids from the junction of exons 18 and 19. DF-1 is a deletion that results in the production of a Rb protein lacking the C-terminal 264 amino-acids.

Transfection of the *RB* wild-type and deletion mutant constructs into COS cells resulted in the synthesis of roughly equivalent quantities of wild-type and aberrant Rb proteins, as determined by immunoprecipitation (Fig. 3a). The mutant proteins are stable, yet are at least partly defective in that they are un- or under-phosphorylated *in vivo* and show reduced (DS2-3 and SS30-1) or no affinity (DF-1) for adenovirus E1A and simian virus 40 (SV40) large T antigen (data not shown). Cotransfection of these mutants with AP-1CAT into 3T3 cells abolished or markedly reduced their ability to repress AP-1 activity (Fig. 3b, lanes 3-6) as compared with the wild-type mouse *RB* (lane 2). Furthermore, repression of *fos* transcription was not detected when the mutants were cotransfected with the FOS-CAT reporter, suggesting that the reduction in AP-1 activity is the result of a reduction in *c-fos* expression by Rb (data not shown). Although we have not examined the nature of the defects in the mutant Rb protein in detail (for example, localization) we conclude from these results that synthesis of wild-type p105-Rb protein is required for the reduction of AP-1 activity and *c-fos* expression.

To map the *cis*-acting element within the *fos* promoter respon-

sible for the Rb repressor effect, progressive 5' deletion mutants of the *fos* promoter were tested for Rb sensitivity in transient cotransfection assays (Fig. 4). A deletion containing only 102 base pairs upstream from the start site of *fos* expression was still repressed by Rb in a cotransfection assay with *RB* (Fig. 4b, lanes 1 and 2), whereas the $\Delta 72$ deletion was not affected (lanes 3 and 4). This analysis delineated an element responsive to Rb transcriptional repression to a 31-nucleotide region between -102 and -71 in the human *c-fos* promoter. To prove that a Rb regulatory element resides between -102 and -71 in the *fos* promoter, a synthetic oligonucleotide was dimerized and inserted directly upstream from the heterologous HSV *tk* promoter in the CAT reporter construct pBL2SVE (Fig. 4a). Plasmid pBL2SVE and the construct containing two copies of the -102 to -71 region of the *c-fos* promoter, pBLRCE (ref. 2), were transfected into 3T3 cells with either the control expression vector or with pmRB. Whereas expression from the *tk* promoter is marginally affected by Rb (lanes 7 and 8), the presence of two copies of the *fos* upstream region significantly increased the sensitivity of the *tk* promoter to Rb-mediated repression (lanes 9 and 10). Thus, an element in the -102 to -71 region of the human *c-fos* promoter is sufficient to confer Rb-mediated transcriptional repression. We have termed this region RCE for retinoblastoma control element.

Our results suggest that Rb can act to downregulate *c-fos* transcription and AP-1 activity. Fos is one component of the heterodimeric transcription factor AP-1 thought to be involved in regulating a set of early response genes required for cell

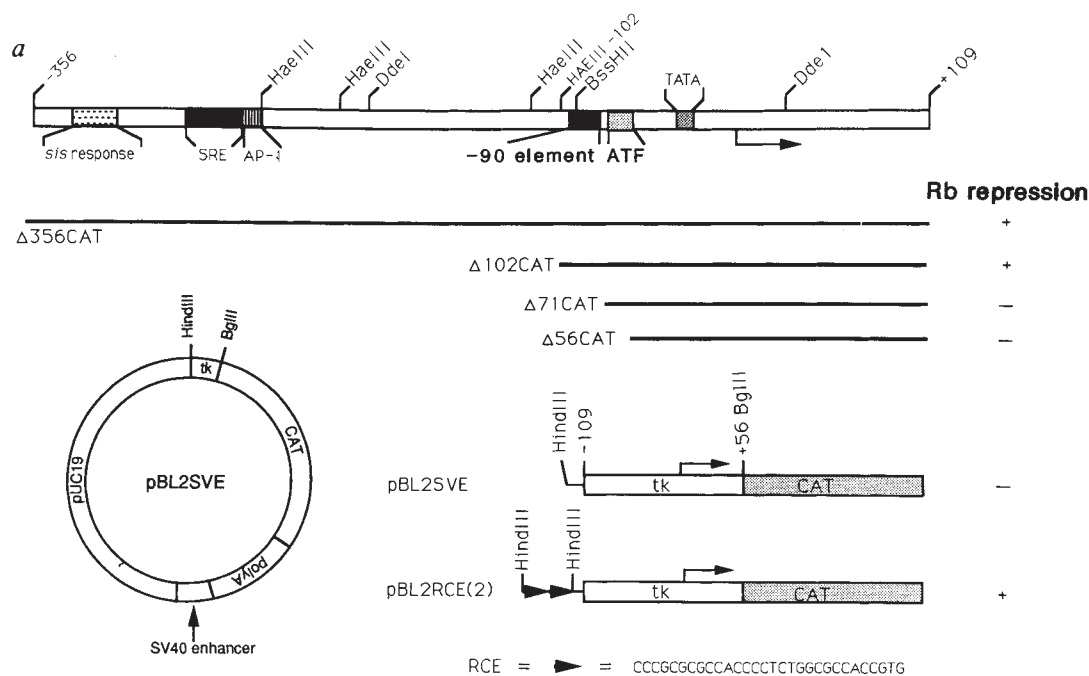
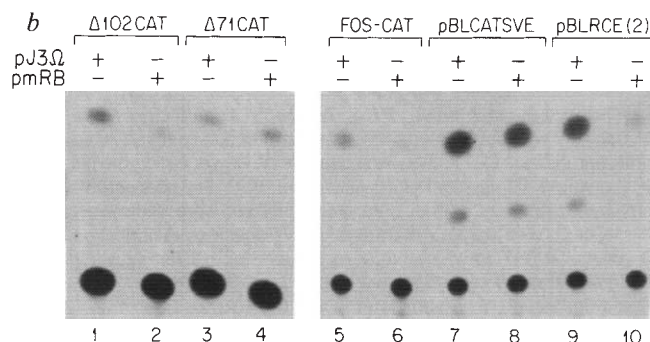


FIG. 4 a, Summary of the mapping of the *cis*-acting element mediating repression by Rb relative to the known regulatory sequences in the *fos* promoter. b, Levels of CAT activity after cotransfection of the appropriate construct with pmRB. The construct $\Delta 102$ CAT was generated by deleting sequences between -356 and a *Hae*III site at position -102; $\Delta 71$ CAT was generated by *Bal*31 digestion¹⁹. The plasmid pBL2SVE was generated by inserting the SV40 enhancer fragment (positions 270-114) into a *Sma*I site 3' to the CAT gene in pBL2CAT. An oligonucleotide containing sequences between positions 102 and -71 (CCCGC-GCGCC-ACCC-CTCTGG-CGCC-ACCGTG) with *Hind*III complementary ends was dimerized and inserted at the *Hind*III site just 5' to the *tk* promoter in pBL2SVE to produce pBLRCE (ref. 2). + in a denotes significant repression by Rb in a cotransfection assay in 3T3 cells.



growth. We presume that a reduction of endogenous *c-fos* expression is directly responsible for the observed reduction in AP-1 transcriptional activity. Rb may also regulate the expression of other components of AP-1 such as c-Jun, JunB and JunD. By downregulating AP-1 activity, Rb could regulate the expression of a set of genes important for cell cycling. Many factors can stimulate *fos* expression through the serum response element in the *fos* promoter, including platelet-derived growth factor, epidermal growth factor, phorbol esters and ionophores^{28,29}. On the basis of our results in both quiescent and cycling cells, we propose that Rb can down-modulate both the level of *fos* stimulation mediated by the serum response element as well as the steady-state level of *fos* expression.

We have mapped the *cis*-acting element conferring repression by Rb to a 31-base-pair sequence lying between -102 and -71 in the *fos* promoter. Previous mutational analysis of the human *fos* promoter has demonstrated that the region around -90 is involved in basal level expression³⁰. Deletion of this region resulted in a 10-fold reduction in the level of *fos* promoter activity when assayed in HeLa cells³⁰. So far we have identified at least two factors from cycling cells that interact with the RCE. How Rb regulates expression from the RCE is not clear; Rb could function by binding directly to the RCE, either alone or in a complex with other cellular proteins. Alternatively, Rb could regulate the activity of a transcription factor(s) and thereby indirectly regulate RCE activity. As we have not so far detected binding of Rb to the RCE, we propose that Rb regulates the activity and/or binding of the factor(s) interacting with the RCE.

SV40 large T antigen and adenovirus E1A can stimulate transcriptional activity through the RCE, suggesting that the element can mediate both positive and negative regulation (P.D.R., J.M.H. and R.C.M., manuscript submitted). This result might have been predicted as both viral proteins bind to p105-Rb (refs 24-26). It is important to note, however, that we have not differentiated between Rb acting to downregulate a positive factor(s) or Rb functioning as a true negative regulator, independently of a positive factor(s). But our results ascribe to the Rb protein a specific function, which might also serve as a paradigm for the activities of other anti-oncogenes. □

Molecular cloning and overexpression of the human FK506-binding protein FKBP

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THE potent immunosuppressive agent FK506^{1,2} is highly effective in preventing organ transplant rejection in humans³. Like cyclosporin A, FK506 inhibits the transcription of early T-cell activation genes⁴, apparently by modulating the activity of transcriptional regulators⁵ such as nuclear factor of activated T cells⁶. A remarkable finding is that the predominant binding proteins (immunophilins) for cyclosporin A and FK506, cyclophilin⁷⁻⁹ and FKBP^{10,11} respectively, are peptidyl-prolyl-*cis-trans*-isomerases that are potently and selectively inhibited by their respective ligands. Here we report the complementary DNA and derived amino-acid sequences of human FKBP from Jurkat cells and also the efficient overexpression in *Escherichia coli* of fully active, recombinant human FKBP. The human FKBP cDNA sequence shows significant similarity to an open reading frame in the *Neisseria meningitidis* genome¹².

A human FKBP (hFKBP) cDNA was cloned by a four-stage polymerase chain reaction (PCR) procedure (Fig. 1a, bottom). In stage 1, PCR with primers designed¹³ from the N-terminal amino-acid sequence¹⁰ was used to obtain 64 base pairs (bp) of explicit nucleotide sequence, which were used in stage 2 to find the remainder of the 3' end of the cDNA by the one-sided PCR procedure¹⁴. The 5'-end of the cDNA was isolated in stage 3 by one-sided PCR amplification of a human peripheral blood acute lymphoblastic leukaemia (HPB-ALL) phagemid library¹⁵, using hFKBP primers and a sequencing primer for the vector. Finally, a full-length cDNA clone was generated with PCR using primers that correspond to the ends of the cDNA.

An open reading frame in the 583-bp cDNA (Fig. 1b) encodes a 108-amino-acid protein in which only the initiator Met₀ precedes the known 19 N-terminal amino acids of hFKBP. Comparison of the cDNA-derived sequence with the sequences of tryptic fragments of bovine FKBP (M. W. Harding, A. G., W. S. Lane and S.L.S., unpublished results) independently confirmed the open reading frame over its entirety; only 3 of 107 residues differ. Although the 5' untranslated region of nine nucleotides is relatively short, the assigned start codon lies within the consensus sequence for translation initiation in eukaryotes (CCRCCATGG)¹⁶. The 247-nucleotide 3' untranslated region possesses a near-consensus CATAAA polyadenylation signal followed by an A_{>25} tail.

Regulated overproduction of FKBP was achieved with the expression-cassette PCR procedure¹⁷, whereby the coding sequence and efficient translation sequences were installed into the expression vector pHN1 (Fig. 1a, bottom). *E. coli* transformed with this construct produced recombinant human FKBP as a significant proportion of soluble cellular protein upon induction (Fig. 2a). N-terminal sequencing and amino-acid analysis confirmed the identity of the recombinant protein and revealed that Met₀ was absent. Natural and recombinant hFKBP are evidently identical, as they co-migrate under isoelectric focusing (pI = 8.8-8.9) and SDS-PAGE (Fig. 2b); consequently, the natural material can be presumed to be free of phosphoryl, glycosyl and acyl substituents. Although mature hFKBP is a protein of relative molecular mass (*M_r*) 11,819, it migrates at

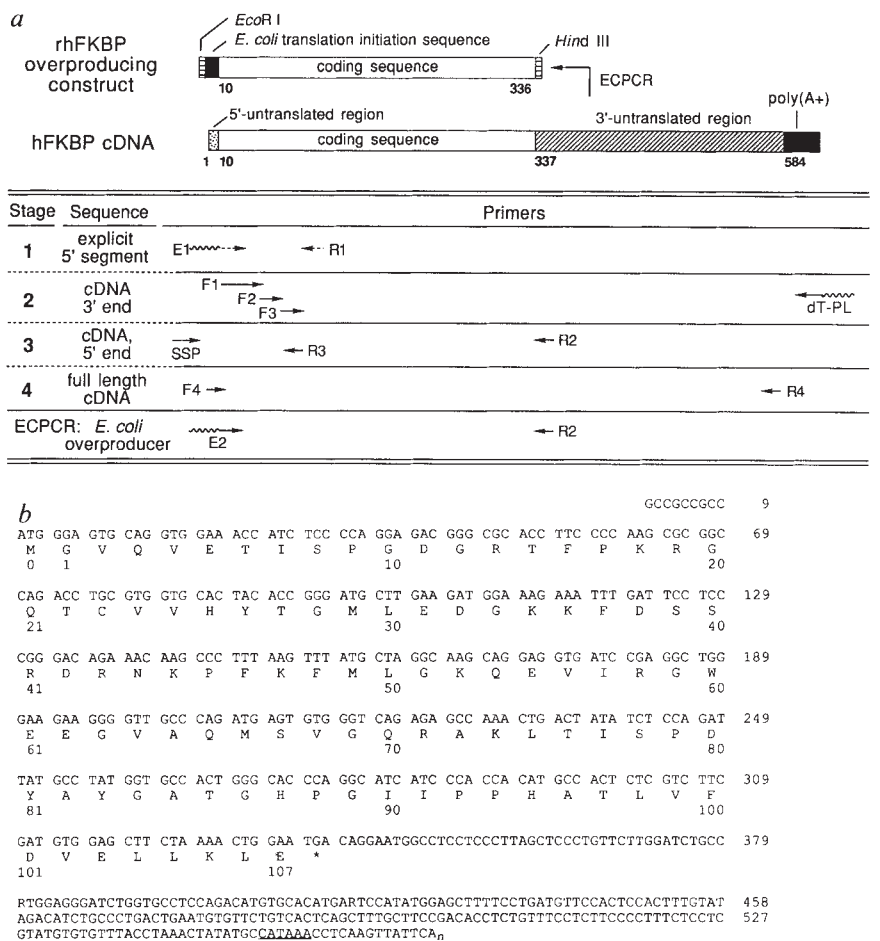
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FIG. 1 Isolation and overproduction of an hFKBP cDNA. **a**, Schematic diagram of hFKBP clones. Top: *E. coli* overproducing construct and cDNA. Below: priming locations for oligonucleotides used in this study. Arrowheads indicate 3'-ends, dashed lines degeneracy, and wavy lines non-hFKBP sequences added for cloning or expression. rhFKBP, recombinant human FKBP; EC-PCR, expression cassette polymerase chain reaction¹⁷. **b**, Nucleotide sequence of the hFKBP cDNA and, immediately below, deduced amino-acid sequence of hFKBP (single-letter code). Asterisk denotes the termination codon, underlining denotes the polyadenylation signal. Two polymorphic sites in the 3' untranslated region are indicated as R (A or G). **METHODS.** A first-strand Jurkat cDNA library was constructed using primer dT-PL (see below) and amplified by PCR²⁹ using primers E1 and R1. The resulting 143-bp fragment was sequenced by asymmetric PCR³⁰. The 3'-end of the cDNA was isolated by the one-sided PCR procedure¹⁴ using F1, F2, and F3, consecutively, with dT-PL. The 5'-end of the cDNA was obtained by one-sided PCR from an HPB-ALL library¹⁵ using R2 and R3 consecutively with SSP (see below), which primes the vector. Two amplification products were generated; **b** shows the longer clone; the shorter lacks the 5'-GCC trinucleotide. No longer PCR product was observed. A full-length cDNA was amplified from the HPB-ALL library using primers F4 and R4, and was sequenced by asymmetric PCR²⁹. The *E. coli* expression cassette was generated using E2 and R2, as described¹⁷, and ligated into pHN1 (H. M. Nash and G. L. V., unpublished results) to give the overexpression construct pHN1-FKBP. Sequences were determined by the dideoxy procedure³¹. Primers were as follows (E, expression; F, forward; R, reverse) (N = A, C, G, T; R = A, G; Y = C, T): E1: ACTATAGGGCGAATTCAGGAGGAATTAATATGGCGTGCAGGTGGARACNAT; E2: ACTATAGGCGGAATTCAGGAGATATACATATGGCGTGCAGGTGGAGACTAT; dT-PL: TCTAGAGTGCAGCTGCAGGCATGCAAGCTT(T)₂₅; PL: TCTAGAGTGCAGCTGCAGGCATGCAAGCTT; R1: TCAAACTTCTTGCCATCYTCNARCAT; R2: TAAGGGAGGAGGCC-ATTCCCTG; R3: CCGGGTGTAGTGCACCAACGCA; R4: GAATACTTGAGGTTTATGCG; F1: GGCCTCCAGGTGGAGACTATCTCCACGAGACGGGCGCACC; F2: ACCT-



TCCTCAAGCGCGCCAG; F3: ACCTGCGTGGTGCACACTACACC; F4: GCGCGCGCATGGGAGTGCA; F5: TCGATGTGGAGCTTCTA; SSP: GCTAAGTAGAGAGAAC-CCAAGT. Primers E1, E2, F1, and F2 differ slightly from the authentic FKBP cDNA sequence. The sequence changes in E2 are silent.

FIG. 2 Induction and purification of recombinant human FKBP (rhFKBP). **a**, Protein composition analysis by SDS-PAGE. Lane 1, Uninduced rhFKBP overproducer, total cell protein; lanes 2 and 3, induced rhFKBP overproducer, total cell protein; lane 4, rhFKBP fractions from DEAE-cellulose chromatography; lane 5, rhFKBP fractions following heat treatment; lane 6, homogeneous rhFKBP obtained from Sephacryl-S100 chromatography; lane 7, hFKBP isolated as described¹⁰; lane 8, bovine FKBP; lane 9, *M_r* markers (pre-stained, Bethesda Research Laboratories). **b**, Comparison of rhFKBP and hFKBP by isoelectric focusing (Ampholine, Pharmacia 1804-101). Lanes 1 and 4, pI markers (Pharmacia); lane 2, rhFKBP; lane 3, hFKBP as in **a**. Proteins in **a** and **b** were stained with Coomassie blue. **METHODS.** *E. coli* XA90 F'lacI^{Q1} (gift of M. Ptashne) transformed with pHN1-FKBP was grown to absorbance $A_{600}=0.6$ at 37 °C in 1 litre of LB medium containing 100 mg ampicillin, and then isopropyl- β -D-thiogalactopyranoside was added to 1 mM. After 10 h, the cells were collected by centrifugation (5,000g for 15 min) and resuspended in 30 ml of lysis buffer (50 mM Tris-HCl (pH 8.0), 2 mM β -mercaptoethanol, 2 mM CaCl_2 , 10 mM MgCl_2 , 5% v/v glycerol). The cells were disrupted in a French pressure cell (4 passages at 1,000 p.s.i.), and the lysate was centrifuged (37,500g for 30 min). The supernatant was loaded onto a 5 $\times$ 70 cm column of DEAE cellulose (Whatman DE-52) equilibrated in packing buffer (PB: 50 mM Tris-HCl, pH 7.5, 2 mM β -mercaptoethanol), and a 500-ml gradient from PB-0.1M KCl was run. Fractions eluting from around 230–300 ml contained rhFKBP. These fractions (15 ml each) were heated to 56 °C for 20 min and centrifuged (3,000g, 30 min). The supernatants were pooled, concentrated to 7 ml (Centri-prep 10, Amicon), and loaded onto a 2.4 $\times$ 100 cm column of Sephacryl-S100 (Pharmacia) equilibrated with 100 mM KCl, 20 mM potassium phosphate (pH 7.5), 0.02% NaN_3 , and 2 mM β -mercaptoethanol. Recombinant hFKBP fractions, which eluted from around 230–260 ml, were pooled and concentrated. N-terminal sequencing found GVQVE of 98% purity (Harvard MicroChem). This procedure typically yields 5–7 mg homogeneous rhFKBP per litre of culture.

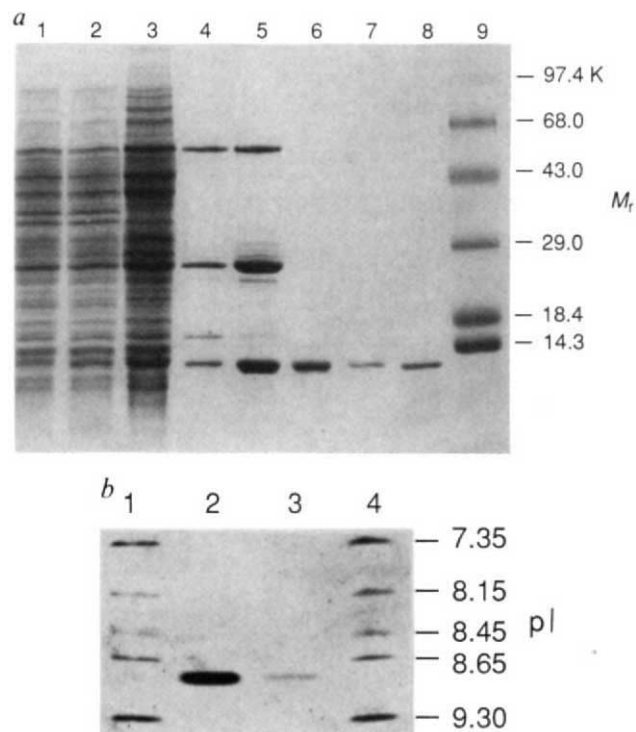
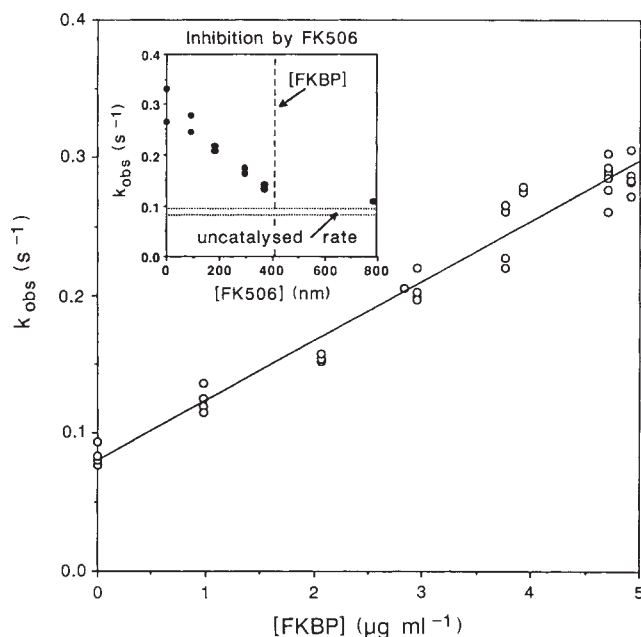


FIG. 3 Peptidyl-prolyl isomerase activity of rhFKBP as a function of concentration. Inset, inhibition of rhFKBP by FK506 as a plot of observed rate constant versus concentration of FK506 for constant protein (410 nM, indicated by the dashed, vertical line); the dashed, horizontal lines mark the bounds of the uncatalysed reaction.

METHODS. The assays were conducted at ambient temperature (26 °C) and followed a variant of the published procedures^{9,11}. Stock protein solution (0.5 mg ml⁻¹) was diluted 10-fold with assay buffer (100 mM Tris-HCl, pH 7.8) and, immediately before assay, further diluted to 970 µl in a 1.5-ml microcentrifuge tube. A 30-µl sample of the peptide substrate (succ-Ala-Ala-Pro-Phe *p*-nitroanilide, 1.7 mM in methanol) was added, and the solution was vortexed and centrifuged for a moment. The reaction was initiated by rapid addition of 970 µl of protein-substrate solution to a 1.5-ml polystyrene cuvette (pre-positioned in the spectrophotometer) containing 30 µl of α-chymotrypsin solution (10 mg ml⁻¹), and absorbance at 410 nm was monitored for 90 s at 0.1-s intervals with a Hewlett-Packard 8452A spectrophotometer. The first-order rate constants were calculated with the KORE program³² (data collected in the mixing period, typically the first 0.5–2 s, were ignored). As the substrate concentration is well below K_m (data not shown), the slope of the line in the plot is k_{cat}/K_m ^{33,34}. When FK506 was used (inset), a methanol solution of the drug (20 µl) and freshly diluted protein (970 µl) were vortexed for 15–20 s and centrifuged for a moment. Peptide substrate (10 µl, 5 mM in methanol) was added, and the assay was completed as above. Protein concentration was calculated from absorbance using $\epsilon^{278}=9,860\text{ M}^{-1}\text{ cm}^{-1}$ (calculated from the aromatic amino-acid content)³⁵. Concentrations determined in this way were found to be within 5% of those determined by amino-acid analysis. Dilutions of a 9.4 mM stock solution of FK506 in methanol were made gravimetrically for accuracy. Dimethylsulphoxide, used elsewhere as the solvent for peptide and drug^{9,11}, was found to inhibit activity and to produce erratic results. Methanol, which has no measurable effect, was therefore used in this study.



about 14,000 (14K) on SDS-PAGE, which resolves the discrepancy in earlier estimates of its molecular weight^{10,11}.

Kinetic analysis showed that the peptidyl-prolyl isomerase activity of recombinant hFKBP is comparable to that of the natural material¹¹ if reasonable allowance is made for difference in temperature and for the different methods of determining protein concentration. (We note that the kinetic data reported in ref. 10 overstate the catalytic activity of hFKBP owing to an error in calculating the protein concentration.) Moreover, FK506 potentially inhibits the enzymatic activity (Fig. 3, inset); at 410 nM protein, one mol-equivalent of drug reduced the catalysed rate by more than 85%, indicating that FK506 and hFKBP form a 1:1 complex. Incubation of recombinant hFKBP with the protease inhibitor phenylmethanesulphonyl fluoride does not affect its activity. The circular dichroism spectrum of recombinant hFKBP (not shown) reveals a predominance of β -sheet (~57%) with little α -helix (~10%)¹⁸, such a composition is reminiscent of the β -fold proteins, which also bind small, hydrophobic molecules¹⁹.

Although FKBP and cyclophilin are both peptidyl-prolyl isomerases, they have no apparent sequence similarity. Furthermore, protein database searches failed to reveal any compelling sequence similarity of FKBP to any known protein. A DNA database search, however, revealed a high degree of sequence identity between the hFKBP open reading frame and a cryptic sequence from the pathogenic bacterium *N. meningitidis* C114 (ref. 12) (Fig. 4). Allowing for a single deletion at position 338 (or 337) in the *N. meningitidis* sequence, the two sequences are 61% identical over the 251-base stretch starting at position 82 of the FKBP cDNA. The sequence similarity, which is high throughout most of the hFKBP coding segment, falls off sharply after the stop codon. Comparison of the predicted, 109-amino-acid translation product of the *N. meningitidis* sequence (allowing for the deletion) with hFKBP reveals that the two are 56% identical over the C-terminal 84 amino acids of hFKBP. Although the similarity is generally weak in the N-terminal region, both open reading frames begin at the same position with a Met-Gly sequence. Closer inspection of the 5' flanking



FIG. 4 Comparison of the hFKBP cDNA sequence with a DNA sequence from *N. meningitidis*. In each row, the sequence (DNA or amino acid) of hFKBP is above that of the bacterium. Derived amino-acid sequences (single-letter code) are below the nucleotide sequences. Vertical bars between DNA sequences denote identity. Open boxes denote amino-acid identity, and stippled boxes conservative changes. A single nucleotide gap was inserted in the *N. meningitidis* sequence at position 338, but could equally well fall at 337 (numbering as in ref. 12). A putative ribosome-binding site (R.B.S.) and promoter in the 5'-region of the *N. meningitidis* sequence are marked by a solid underline; the dashed overbars mark two sequences that resemble the -35 and -10 consensus sequences but that have a non-consensus spacer. The NBRF protein database (release 35.0, 6/89) and the Genbank (release 60.0, 6/89) and EMBL (release 19.0, 5/89) nucleic acid databases were searched using the GCG software package (University of Wisconsin)³⁶.

sequence in *N. meningitidis* reveals a promoter²⁰ and a ribosome-binding site²¹ that closely match the consensus sequences, suggesting that the *N. meningitidis* sequence is expressed; however, the C114 strain encodes a protein probably disabled by the deletion-induced frameshift.

The role of FKBP in human T-cell activation is unknown, as is the relevance of its peptidyl-prolyl isomerase activity. Cyclophilin has been shown to mediate the biological effects of cyclosporin A in two lower eukaryotes²²; by analogy, it is likely that FKBP mediates the biological effects of FK506, notably its selective inhibition of calcium-dependent²³ lymphokine gene transcription⁴. As FKBP lacks the leucine zipper, helix-turn-helix, zinc finger²⁴ and activation domain²⁵ motifs, it is probably not a direct activator of transcription. Likewise, FKBP lacks a calcium-binding motif²⁶, so its own function is probably not affected by calcium. A plausible model is that an FKBP, like the cytoplasmic anchoring proteins²⁷, binds a component of the transcriptional apparatus and thereby controls the activity of transcription factors. FK506, bound to the FKBP, might for example prohibit release by blocking a phosphorylation site that is required to effect dissociation. A unifying mechanism that explains the inhibitory effects of FK506 (and cyclosporin A)⁶ on a family of transcription factors calls for the interaction of the immunophilin with an as-yet-unknown pleiotropic factor²⁸ that activates the transcription activators further along in the signal transduction pathway, such as the nuclear factor of activated T cells and AP-3 (ref. 6). □

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Isolation and sequence of an FK506-binding protein from *N. crassa* which catalyses protein folding

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SLOW protein-folding reactions are accelerated by a prolyl *cis/trans* isomerase isolated from porcine kidney^{1,2} which is identical^{3,4} to cyclophilin⁵, a protein that is probably the cellular receptor for the immunosuppressant cyclosporin A^{6,14}. Catalysis probably involves the isomerization of prolyl peptide bonds in the folding protein chains. Cyclosporin A inhibits folding catalysis by cyclophilin⁴. Here we report the isolation, cloning, sequencing and expression of another protein with prolyl isomerase activity from *Neurospora crassa* which is unrelated to cyclophilin and which also catalyses slow steps in protein folding. This protein does, however, show sequence similarity to a human protein⁷⁻⁹ that binds to another, recently discovered immunosuppressive drug, FK506 (ref. 10). Moreover, it shares 39% identity with the carboxy-terminal 114 residues of a cell-surface protein from the bacterium *Legionella pneumophila*, the causative agent of Legionnaires' disease^{11,12}. Catalysis of folding by the FK506-binding protein from *N. crassa* is inhibited by FK506, but not by cyclosporin A. Thus, at least two different classes of conformationally active enzymes

(conformases¹³) exist that catalyse slow steps in protein folding. Both occur in a wide variety of cells and are inhibited by immunosuppressive drugs.

N. crassa mutants that have completely lost the cyclophilin gene product still contain residual prolyl isomerase activity (ref. 14, and unpublished results). During the isolation of cytosolic cyclophilin^{15,16} from wild-type *N. crassa*, a second protein with prolyl isomerase activity co-purified in the initial steps, migrating with an apparent relative molecular mass of 14,000 (M_r 14K) on SDS-PAGE (Fig. 1, lane 3). We purified this protein to apparent homogeneity (Fig. 1, lanes 4 and 5). Like cyclophilin, it is abundant, comprising 0.1-0.2% of cytosolic protein.

The protein is active as a prolyl isomerase and is inhibited by the novel immunosuppressant FK506. We used the method of Fischer *et al.*¹ to determine the prolyl isomerase activity of the *N. crassa* FK506-binding protein (Nc-FKBP). In this assay the rate of the *cis* → *trans* isomerization of the Ala-Pro bond in the synthetic peptide *N*-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide is measured in the absence and in the presence of a putative prolyl isomerase. Nc-FKBP is about 20% as active as cyclophilin from the same organism in this assay (Fig. 2a). Unlike cyclophilin⁴, but like human FKBP^{7,8}, Nc-FKBP is not inhibited by cyclosporin A (CsA) at concentrations as high as 10 μ M. Inhibition of prolyl isomerase activity by FK506 is, however, observed in the nanomolar range (Fig. 2b).

Nc-FKBP catalyses slow, rate-determining steps in protein folding. We used the folding of ribonuclease T1 from *Aspergillus oryzae* as a model for two reasons. First, its slow folding is thought to involve steps that are controlled by proline isomerization¹⁷ and, second, catalysis by porcine cyclophilin of the two principal slow-folding phases of this protein has been shown previously^{4,18}. Nc-FKBP catalyses the two slow phases of RNase T1 folding, albeit with different efficiencies (Fig. 3a). Catalysis of protein folding is generally less efficient than acceleration of proline isomerization in the assay peptide. Nc-FKBP at 1.34 μ M

leads to a 10-fold increase in folding rate (faster phase) of RNase T1, but to a 100-fold acceleration of isomerization of the synthetic peptide. Part of this difference probably originates from restricted accessibility of the Xaa-Pro bonds in the partially folded protein, where Xaa is any amino acid. Another factor is that a *cis* → *trans* isomerization is catalysed in the assay peptide, whereas *trans* → *cis* interconversions are rate-determining in RNase T1 folding. Under the conditions of Fig. 3a, cyclophilin from *N. crassa* is about threefold more efficient as a folding catalyst; cyclophilin from porcine kidney shows a 15-fold higher activity (E.R.S., S.M. & F.X.S., unpublished results). Catalysis of RNase T1 refolding by Nc-FKBP is inhibited by FK506. The activity of 0.67 μ M Nc-FKBP is decreased by 95% in the presence of 1 μ M FK506 (Fig. 3b). Unlike porcine cyclophilin, Nc-FKBP is not inhibited by 1 μ M cyclosporin A in its catalysis of protein folding (Fig. 3b).

A full-length complementary DNA coding for Nc-FKBP was cloned. Polyclonal antisera raised against the isolated protein (Fig. 1, lanes 4 and 5) were used to screen an *N. crassa* pEX expression library^{19,20}. To confirm the identity of the longest insert (Fig. 4a), it was cloned into the pGEM4 transcription vector²¹. Transcription *in vitro* of the resulting plasmid, either intact or cut with the restriction enzyme *Nsi*I (see Fig. 4a), and subsequent translation of the RNAs in reticulocyte lysates^{22,23} gave in both cases authentic Nc-FKBP which could be immunoprecipitated by specific antisera and which comigrated with the isolated protein on SDS-PAGE (not shown).

The sequence of the full-length cDNA insert and the deduced amino-acid sequence of Nc-FKBP are shown in Fig. 4a. Nc-FKBP is a weakly basic protein with M_r 13,022, which is unrelated to cyclophilin in sequence. Unlike cyclophilin, Nc-FKBP has no cysteine residues. The amino terminus of Nc-FKBP shows high sequence similarity to the recently published amino terminus of human FKBP^{7,9} (Fig. 4b). We conclude that we have isolated and cloned the *N. crassa* homologue to human FKBP. A protein database search (MIPSX, release 15-0, 23 January 1990) revealed only one sequence similarity to another protein, that is, to the carboxy-terminal part of a cell surface protein from the pathogenic bacterium *Legionella pneumophila*, the

causative agent of Legionnaires' disease^{11,12} (Fig. 4c). This protein (mip), of M_r 24,000, is associated with macrophage infectivity by an unknown mechanism^{11,12}. It is not known whether this protein binds FK506 or is active as a prolyl isomerase. Interestingly, this FKBP-related protein occurs in a membrane-associated form. Similarly, a protein of the cyclophilin family,

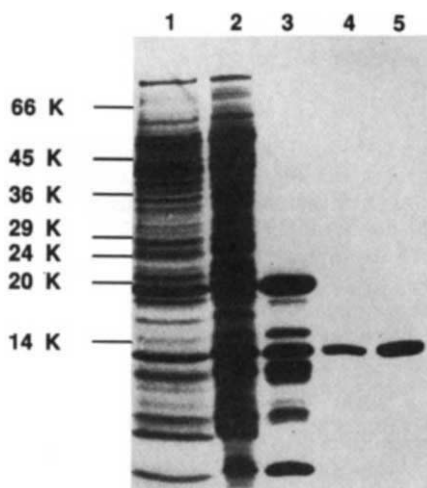


FIG. 1 Purification of Nc-FKBP. Starting with *N. crassa* cytosol (lane 1; 100 μ g)¹⁵, proteins were passed over a 100K-exclusion membrane²⁶. The flow-through (lane 2; 100 μ g) was passed over a 10K-exclusion membrane (Millipore). A few proteins were enriched in the 10K retentate (lane 3; 50 μ g), one of which is cyclophilin (20K)¹⁵; Nc-FKBP with an apparent M_r of 14K (lanes 4, 5 μ g; and 5, 10 μ g) was purified to apparent homogeneity using a Fractogel TSK HW-50 (S) molecular sieve column (1 $\times$ 150 cm, 1 ml h⁻¹ flow rate in 10 mM MOPS buffer, pH 7.5, 50 mM KCl, 0.1 mM DTT). The various fractions were subjected to SDS-PAGE and protein bands were stained with Coomassie Brilliant Blue.

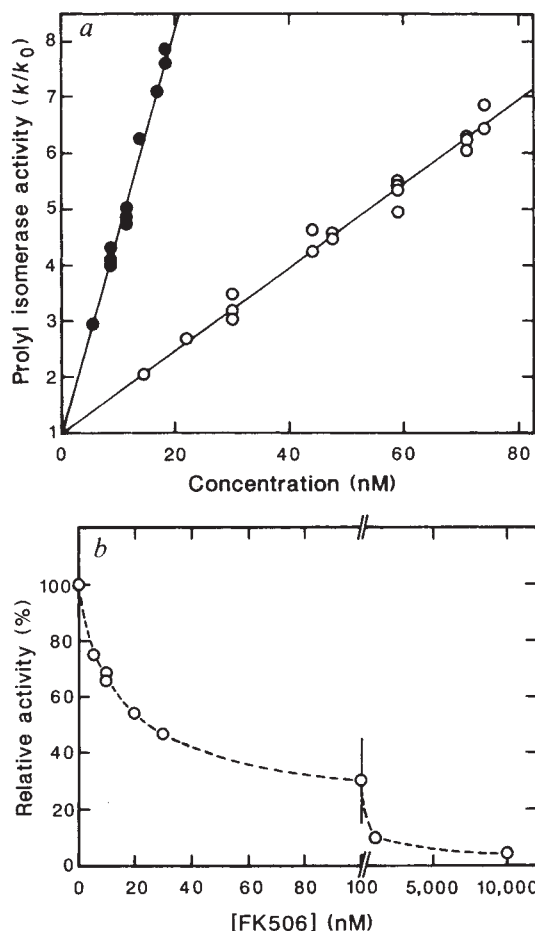


FIG. 2 Prolyl isomerase activity of Nc-FKBP. a, Activity as a function of Nc-FKBP concentration ($\circ$) and comparison with that of cytoplasmic cyclophilin ($\bullet$) from *N. crassa*. b, Inhibition by FK506 of the prolyl isomerase activity. Activity is shown as k/k_0 , where k and k_0 are the rate constants of isomerization in the presence and in the absence of isomerase activity, respectively. Under the assay conditions (10 °C, pH 8.0) $k_0 = 7.75 \times 10^{-3} \text{ s}^{-1}$. METHODS. The *cis* → *trans* isomerization of the Ala-Pro peptide bond in the assay peptide *N*-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide (Sigma) was measured in a coupled assay with chymotrypsin (Boehringer), on the basis of the ability of this protease to cleave the assay peptide only when the Ala-Pro bond is in the *trans* conformation¹⁴. Nc-FKBP was equilibrated for 15 min in 0.1 M Tris-HCl, 1 mM EDTA, pH 8.0 in the spectrophotometer cell at 10 °C. Then α -chymotrypsin (21 μ M) was added, and the assay was started 20 s later by adding 30 μ l of a stock solution of the assay peptide (2.3 mM in dimethylsulphoxide). During the reaction the enzyme activity is not affected by dimethylsulphoxide. The final volume was 1.0 ml, the final concentration of assay peptide was 70 μ M. The *trans* peptide was cleaved within the deadtime of mixing ($\sim$ 5 s); hydrolysis of the 4-nitroanilide in the *cis* peptide is limited in rate by the *cis* → *trans* isomerization at Ala-Pro and was followed by the increase in absorbance at 390 nm. Cytoplasmic cyclophilin from *N. crassa* was isolated as described¹⁵. The concentrations of Nc-FKBP and cyclophilin were determined from their absorbance at 280 nm. The absorption coefficients (cyclophilin, $\epsilon_{280} = 16,500 \text{ M}^{-1} \text{ cm}^{-1}$; Nc-FKBP, $\epsilon_{280} = 9,530 \text{ M}^{-1} \text{ cm}^{-1}$) were calculated from the content of aromatic residues²⁷. In all cases the substrate concentration was much smaller than K_m , therefore there was no deviation from first-order kinetics in the assays. In the inhibition experiments in b, Nc-FKBP (48 nM) was preincubated for 15 min with varying concentrations of FK506 and the remaining prolyl isomerase activity determined.

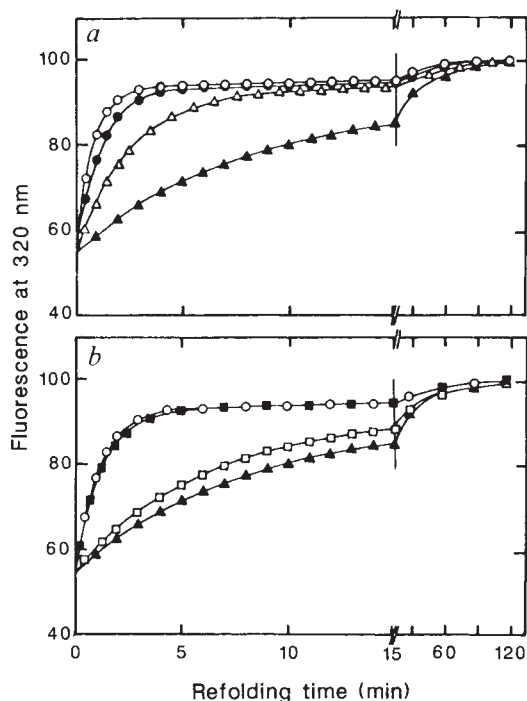


FIG. 3 Catalysis of protein folding by Nc-FKBP. *a*, Slow refolding kinetics of RNase T1 in the presence of increasing amounts of Nc-FKBP. The change in fluorescence at 320 nm is shown as a function of the time of refolding. The final conditions for refolding were 2.5 μM RNase T1 in 0.2 M urea and 0.1 M Tris-HCl, pH 8.0, 1 mM EDTA at 10 °C. The refolding solution contained the following concentrations of Nc-FKBP, (▲) 0 μM; (△) 0.34 μM; (●) 0.67 μM; (○) 1.34 μM. The observed kinetics were analysed in terms of a sum of two first-order processes¹⁷. The resulting time constants and corresponding amplitudes are $\tau_1 = 3,000$ s ($A_1 = 0.19$), $\tau_2 = 500$ s ($A_2 = 0.38$) at 0 μM Nc-FKBP; $\tau_1 = 2,700$ s ($A_1 = 0.11$), $\tau_2 = 140$ s ($A_2 = 0.48$) at 0.34 μM Nc-FKBP; $\tau_1 = 2,300$ s ($A_1 = 0.09$), $\tau_2 = 60$ s ($A_2 = 0.46$) at 0.67 μM Nc-FKBP; $\tau_1 = 2,250$ s ($A_1 = 0.10$), $\tau_2 = 45$ s ($A_2 = 0.51$) at 1.34 μM Nc-FKBP. The amplitudes (A_1 , A_2) are given as fractions of the final fluorescence, observed after complete refolding. A similar decrease in amplitude of the slowest phase was also observed in the presence of porcine cyclophilin as a folding catalyst¹⁷. *b*, Effect of FK506 and of cyclosporin A on the catalysis by Nc-FKBP of the folding of RNase T1. Uncatalysed folding in the absence of Nc-FKBP (▲). Folding kinetics in the presence of 0.67 μM Nc-FKBP (○) and of additional 1 μM FK506 (□) and of 1 μM cyclosporin A (■), respectively. METHODS. Recombinant RNase T1 was a gift from U. Hahn (Berlin). Unfolded protein was generated by a 2-h incubation of 100 μM RNase T1 in 8.0 M urea, 0.1 M Tris-HCl and 1 mM EDTA, pH 8.0 at 25 °C. Refolding was initiated by diluting 25 μl unfolded protein with 975 μl 0.1 M Tris-HCl, 1 mM EDTA, pH 8.0, containing the appropriate concentrations of Nc-FKBP, FK506, and cyclosporin A in the fluorimeter cell at 10 °C. The final conditions for refolding were 2.5 μM RNase T1 in 0.2 M urea, 0.1 M Tris-HCl, 1 mM EDTA, pH 8.0, at 10 °C. The refolding reaction was monitored by the increase in fluorescence at 320 nm (10 nm bandwidth) after excitation at 268 nm (1.5 nm bandwidth) using a Hitachi 4020 fluorescence spectrophotometer.

the *ninaA* gene product of *Drosophila melanogaster*, is presumably membrane-bound^{24,25}.

The FK506-binding protein from *N. crassa* represents a further example of an enzyme that can accelerate slow steps in protein folding. The characterization of a FK506-binding protein in a lower eukaryote shows that this class of proteins is not restricted to cells of the immune system, where the drug FK506 has been proposed to act as an immunosuppressive agent and where the binding protein was initially discovered^{7,8}. The two classes of receptors for immunosuppressants, FKBP and cyclophilins, are structurally distinct, they are widely distributed in unrelated organisms, and they share a common activity as catalysts of slow steps in protein folding *in vitro*.

The cellular functions of prolyl isomerases are still unknown. We suggest that they act *in vivo* on proline-containing chain

a

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-55      GTACACCACTCTCTCAAAACAAACCAACCACTTCAACCAACATCCAGCAG
1  ATGACTATTCCCGAGCTTGACGGTCTTCAGATCGAGGTCCAGCAGGAGGCCAGGGCACC
1  M T I P Q L D G L Q I E V Q Q E G Q G T

61  CGTGAGACCGCTGCGGGGACAACTGACGCTCCACTACAGGGTGTCTCACCACGGT
21  R E T R R G D N V D V H Y K G V L T S G

121 AAGAAGTTCGACGTAGCTACGACGCTGGCGAGCTCTCAACTTCACGGTGTGGCAGGGC
41  K K F D A S Y D R G E P L N F T V G Q G

181 CAGTTCATCAAGGGCTGGGATGAGGCTCTCTCGGCATGAAGTGTGGCAGAGCGGAGG
61  Q V I K G W D E G L L G M K I G E K R K

241 CTCATATCGCCCTCACTCGCTCAAGGCAACCCCGCGCTGGTGGCATCATCCCCGCC
81  L T I A P H L A Y G N R A V G G I I P A

301 AACTCACCTCATCTTUGAGACCGAGCTCGTGGTATCAAGGGTTCAGAGCGGAGG
101 N S T L I F E T E L V G I K G V Q K G E

361 TAAATGGATCACTTACAAATGCACTATAGCTCTGGGATACAGGGGATTAACGGGTCAA
421 TGAAGATCATTTTCGGGCTTATGCTCTTAACGGTGTGACTGCGCTGAGCGCTTCTC
481 GAGTCGAGATTGGGGCTGGACAGATCTATTTCAGGGTCATATTAGGAGGAAACGTT
541 GCATAAAGACAGACCGGCTGGCTGCTAAAAATAAAAAAAAAA
  
```

b

```

N.c. 1 MTIPQLDGLQIEVQEGCGGTRET--RRGDN-VDVHYKGVLTSGKKFDASYDRGEPLNFTV
H.s. 1 GVQVETISPGDG-R-TFGKRGQTCV-VHYTGMLDGKKFDSRDRKRFHML
  
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c

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FKBP 1 MTIPQLDGLQIEVQEGCGGTRETTRGDNVDVHYKGVLTSGKKFDASYDRGEPLNFTVQ
mip 120 GVVLPSSGLQYKVINAGNGVKGK-SDVTVEYTGRLIDGTVDSTETKGPATFCVSSQ--

FKBP 62 VIKGWDEGLLGKIKGKRLTIAPHLAYGNRAVGGIIPANSTLIFETELVGIKGVQKGE
mip 178 VIPGWTEALQLMPAGSTWEIYVPSGLAYGPRSVGGPIGNETLIFKTHLISVKKSS
  
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FIG. 4 *a*, cDNA and derived amino-acid sequence (single-letter notation) of Nc-FKBP. The longest insert identified by immunoscreening an *N. crassa* pEX expression library^{19,20} was subcloned in pGEM4 (Promega)²⁸ either intact or as restriction fragments using internal *Rsa*, *Sal*, *Sac* or *Nsi* sites (underlined). A putative polyadenylation signal (ATAAAA) is also underlined. The cDNA was sequenced using SP6 or T7 primers and Nc-FKBP-specific primers. Double-strand sequencing²⁹ was performed according to the dideoxy termination method³⁰ using ³⁵S-labelled dATP (ref. 31) and Sequenase 2.0 (USB)²⁸. Both strands were sequenced at least three times. *b*, Comparison of the N-terminal sequences of human (H.s.)^{8,9} and *N. crassa* (N.c.) FKBP. The first 50 residues of the human protein were aligned with the N-terminal 57 amino acids of the *N. crassa* protein. *c*, Sequence similarity of Nc-FKBP to the C-terminus (residues 120–233) of the cell-surface protein mip of *L. pneumophila*^{11,12}. In *b* and *c* identical amino acids are shown in bold. Gaps were introduced to maximize homology.

regions of other proteins. Their targets could be folding nascent polypeptide chains, unfolded proteins which have traversed a biological membrane, and/or folded proteins with accessible prolyl peptide bonds. Preliminary experiments (M.T. *et al.*, unpublished results) indicate that, like cyclophilin¹⁵, an FK506-binding protein is present in *N. crassa* mitochondria as well. Delayed folding in the absence of prolyl isomerase could lead to decreased levels of regulatory proteins in the cell, thus explaining some of the effects of cyclosporin A and FK506. Catalysis of proline isomerization in folded proteins has not been found so far, but isomerization is an intrinsically slow process in folding polypeptides as well as in native proteins. Thus, it might serve as a slow switch between alternative (such as active and inactive) conformations of a target protein.

Note added in proof: We learned that Dr S. Schreiber's group (Harvard) has cloned the human FK506-binding protein. It shares 50% sequence identity with Nc-FKBP. □

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Abrupt changes in flagellar rotation observed by laser dark-field microscopy

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BACTERIA such as *Escherichia coli* and *Salmonella typhimurium* swim by rotating their flagella^{1,2}, each of which consists of an external helical filament and a rotary motor embedded in the cell surface (see ref. 3 for a review). The function of the flagellar motor has been examined mainly by tethering the flagellar body to a glass slide and observing the resultant rotation of the cell body². But under these conditions the motor operates at a very low speed

(about 10 r.p.s.) owing to the unnaturally high load conditions inherent in this technique. Lowe et al.⁴ analysed the frequency of light scattered from swimming cells to estimate the average rotation speed of flagellar bundles of *E. coli* as about 270 r.p.s. To analyse motor function in more detail, however, measurement of high-speed rotation of a single flagellum (at low load) with a temporal resolution better than 1 ms is needed. We have now developed a new method—laser dark-field microscopy—which fulfils these requirements. We find that although the average rotation speed of *S. typhimurium* flagella is rather stable, there are occasional abrupt slowdowns, pauses and reversals (accomplished within 1 ms). These changes were frequently observed in mutants defective in one of the motor components (called the switch complex), suggesting that this component is important not only in switching rotational direction but also in torque generation or regulation.

Individual flagellar filaments can be observed on cells stuck to a glass slide. (The load for the motors of a stuck cell is

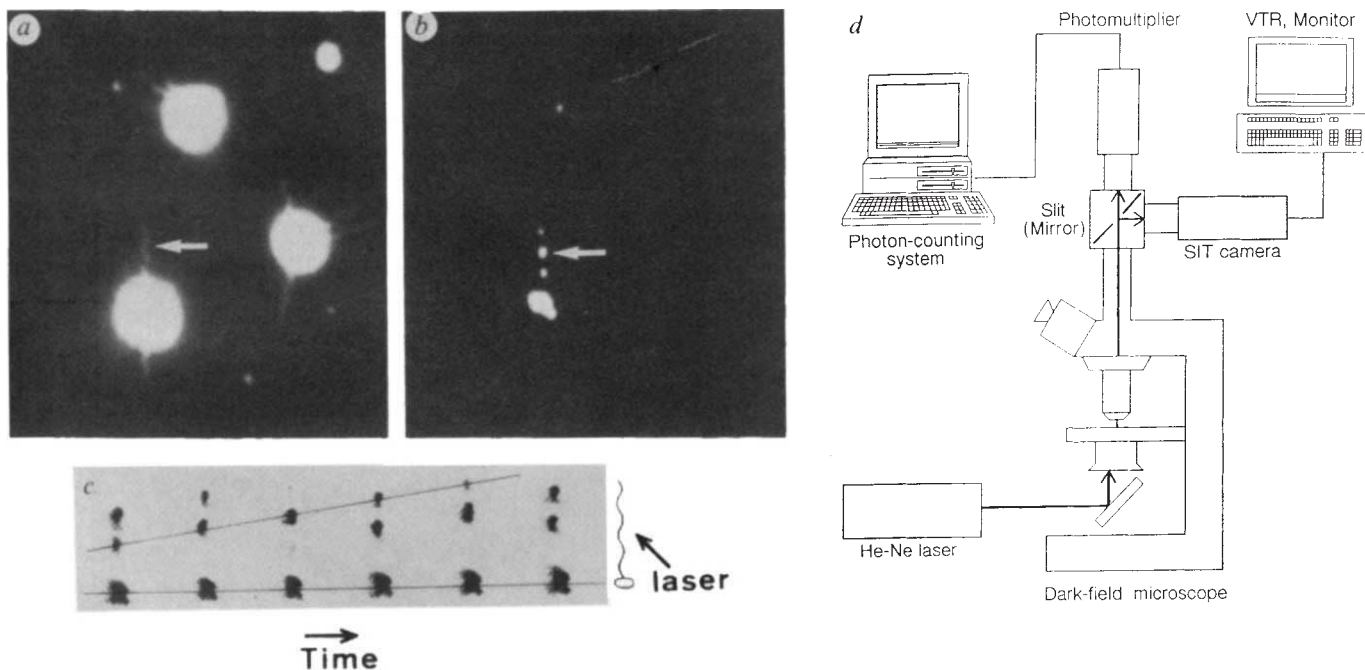


FIG. 1 Dark-field images of bacteria and schematic diagram of the experimental set up. *a*, Ordinary dark-field image of *S. typhimurium* (flagella stopped). *b*, Laser dark-field image of the same field as in *a*. Arrows in *a* and *b* indicate the same filament. The laser path is from the lower right to the upper left of *b*. The parts of the (helical) filament illuminated obliquely are dark, whereas those illuminated approximately normally are bright. The image of the cell body with the illuminated filament is not as bright as in *a*, and the other two cells are not seen, because the diameter of the laser beam is very small (5–6 μm). Photodamage of the cell is minimal under this illumination condition (see legend to Fig. 2). *c*, Sequential images of a slowly rotating, laser-illuminated flagellum (negative images; video recording was possible only because of the slow rotation). *d*, Schematic diagram of the

measuring system. An image of a flagellum is focused on a slit located on a mirror plate. The intensity of light passing through the slit is detected and recorded by a photon-counting system. The images of the flagellum and the slit are simultaneously monitored with a video system, so that the position of the flagellar image can be adjusted onto the slit. The instruments used were: a He-Ne laser (NEC, GLG5800), a dark-field condenser (Olympus, DCW 1.4–1.2), an objective lens (Nikon, Plan 40 $\times$, NA 0.70), a relay lens (Nikon, TV 1 $\times$), a photomultiplier tube (Hamamatsu Photonics, R649), a photon counting unit (Hamamatsu Photonics, C1050), a counter (Yatoro Inc.), a computer (NEC, PC9801vm), an SIT camera (Hamamatsu Photonics, C2400-08) and a video recorder (Victor, HR-S7000).

FIG. 2 Measured intensity (I) changes and calculated rotation speed. The data were obtained using a *che*-deletion mutant of *S. typhimurium*, Δche (see below), at room temperature. *a*, An example of raw data. Part of a 30-s measurement, with a photon-counting gate time of 400 μ s, is shown. *b*, Another part of the data with an expanded timescale. The positions of speed changes are indicated by arrows and the period of slower rotation (S) is indicated by the horizontal bar. The positions of speed changes were detected by changes in slope and were determined by comparing each peak's profile with those of its neighbours. *c*, Rotation speed as a function of time (right) and the FFT (fast Fourier transformation) spectrum of intensity changes (left), where 'PD' is the probability distribution of rotation speed. Rotation speeds shown on the right were calculated from peak intervals in the raw data; they are the speeds averaged within each cycle of rotation (R_{av}). The main error in determining R_{av} derives from the error in measuring peak positions ($\sim 6\%$ at 150 r.p.s.). *d*, Intensity of light scattered from one edge of a cell body which had rotating flagella. This demonstrates the noise level of the measuring system. The intensity of illuminating laser light was decreased to 1/1,000 using neutral density filters, so that the photon counting rate was at a level similar to that of a flagellum. Strains: Proteins of the chemotactic (*Che*) system modulate the function of the switch complex, which consists of three proteins and is thought to control the switching of the motor between counter-clockwise (CCW) and clockwise (CW) rotation^{1,3}. To simplify the analysis, we used *che*-deletion mutants of *S. typhimurium* whose flagellar rotation was strongly CCW-biased. Two groups of such *che*-deletion strains were examined: (1) Δche , SJW3076, *cheA*-*cheZ* deleted, and (2) *sw*/ Δche , Δche with an additional mutation in one of the switch genes. These *sw*/ Δche mutants were derived from pseudorevertants of a *cheY* mutant, SJW2903, and the mutation numbers are indicated in parentheses (for details of mutant isolation and characterization, see ref. 15). METHODS. Cells were grown at 37 °C in Luria broth, collected at late-log phase, washed once and suspended in 20 volumes of motility medium (10 mM potassium phosphate, pH 7.0, 10 mM glucose, 10 mM NH_4Cl , 1 mM cysteine, 0.01% yeast extract, and 0.1 mM EDTA). This suspension (3.5 μ l) was put on a glass slide, covered, and sealed with silicone oil. More than half of the cells stuck to the slide or cover glass. By using a stuck cell, each flagellar filament could be observed separately for a long time even in the case of a peritrichously flagellated bacterium such as *S. typhimurium*, because a flagellar bundle is not formed. The slide was left at room temperature or at 30 °C for 1 h to make cells anaerobic before observation at the same temperature. Cells of *S. typhimurium* under anaerobic conditions were more resistant to intense light than those under aerobic conditions: most flagella on the former cells could rotate for longer than 1 h, whereas those on the latter stopped in a few minutes.

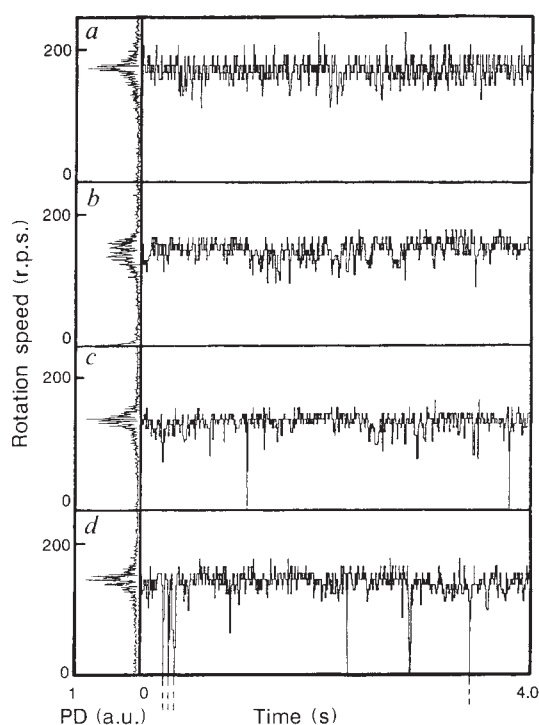
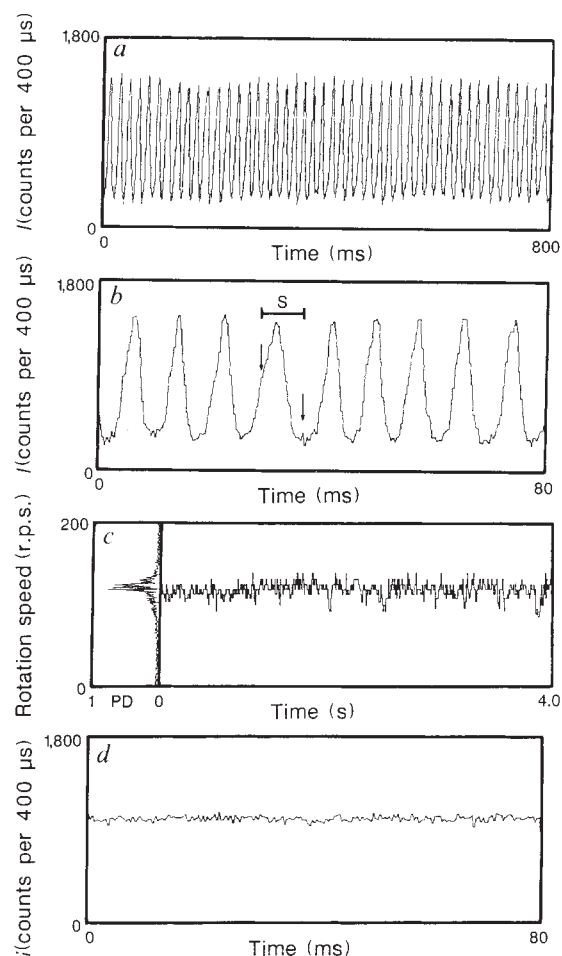


FIG. 3 Rotation speeds, R_{av} (right, see legend to Fig. 2 for definition) and FFT spectra of intensity changes (left). The data were obtained using strains of *S. typhimurium* at 30 °C. *a*, Δche ; *b*, *sw*/ Δche (15); *c*, *sw*/ Δche (37); *d*, *sw*/ Δche (8) (see legend to Fig. 2). The broken lines in *d* indicate reversed rotations. The occurrence and severity of speed changes such as slowdowns, pausings and reversals varied among strains. This excludes the possibility that the speed changes observed here were artefacts, such as results of instantaneous contacts between the glass surface and the flagellar filament during its rotation.

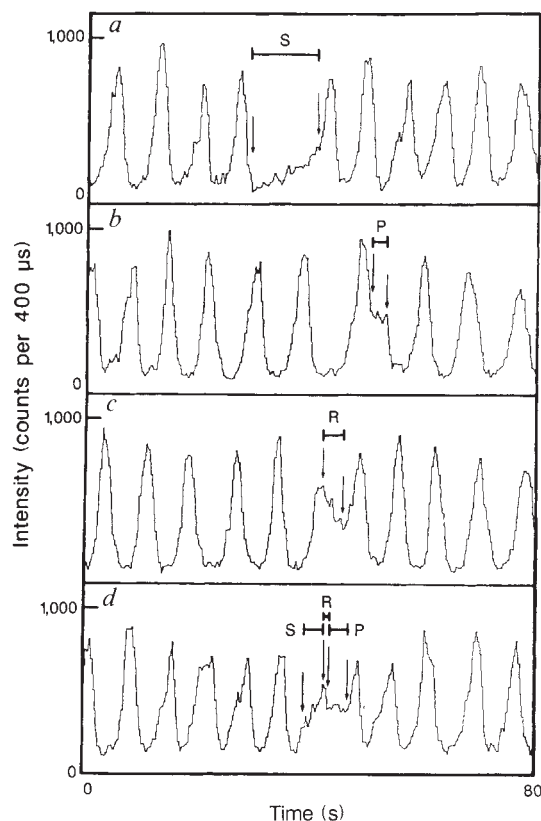


FIG. 4 Examples of speed changes observed in *sw/Δche* (8) at 30 °C. The positions of speed changes are indicated by arrows and the periods of slower and reverse rotation and pausing are indicated by horizontal bars. S, slower rotation; P, pausing; R, reversed (CW) rotation.

presumed to be almost the same as that for the motors of a swimming cell^{5,6}). By ordinary dark-field illumination^{7,8}, the image of a helical flagellar filament appears as a sinusoidal line (Fig. 1a). On the other hand, by one-sided laser illumination, the image of a helical filament appears as a series of bright spots, with one spot per period of the helix (Fig. 1b). As the flagellum rotates, the array of bright spots moves along the helical axis (Fig. 1c). The image of the flagellum is then focused on an optical slit, and the intensity of light passing through the slit is measured (Fig. 1d). Alternating increases and decreases of light intensity are observed (for example Fig. 2a); each cycle corresponds to one flagellar rotation. Bright illumination by a focused laser beam and a photomultiplier with sufficiently high sensitivity and small time constant allow measurement with a temporal resolution of about 1 ms.

The rotation of single flagella of numerous *che*-deletion strains of *S. typhimurium* was examined: one strain with a wild-type switch complex (*Δche*) and 50 strains with mutant switch complexes (*sw/Δche*, see legend to Fig. 2).

The results obtained with *Δche* at room temperature are shown in Fig. 2. The average flagellar rotation speed (R_{av}) was around 115 r.p.s. The fluctuation (s.d.) of R_{av} (the speeds averaged within each cycle of rotation) was only 7.7% (Fig. 2c). This long-term stability in rotation speed is also seen with tethered cells¹⁴. Abrupt changes in rotation speed were accomplished within the time corresponding to two gate-time units of photon counting (0.8 ms). With the high temporal resolution of this method, such speed changes were detected as changes in slope (Fig. 2b). The magnitude of the slowdown in Fig. 2b was estimated to be 35%, and the slower rotation lasted only 8 ms. The noise level shown in Fig. 2d was sufficiently small to ensure that the abrupt speed changes were detectable (Fig. 2b; see also Fig. 4).

Figure 3 shows R_{av} for *Δche* and three typical *sw/Δche* strains at 30 °C as a function of time. The rotation speed of *Δche* was around 170 r.p.s.; this is slower than the bundle frequency of 270 r.p.s. in *E. coli* at 32 °C observed by Lowe *et al.*⁴, probably because our experiments were carried out under anaerobic conditions whereas theirs were under aerobic conditions⁹. Although

R_{av} of *Δche* was stable, with a fluctuation (s.d.) of 9.5% (Fig. 3a), R_{av} of the *sw/Δche* strains was less stable, with fluctuations (s.d.) of 10.6, 11.3 and 14.0% (Fig. 3b, c, d). Frequent slowdowns, pauses and reversals were observed (Fig. 3b, c, d; see also Fig. 4).

Figure 4 shows examples of raw data obtained for one of the *sw/Δche* strains. Changes in rotation speed, including slowdowns (~75% drop in rotation speed in Fig. 4a), pauses and reversals, were accomplished within 1 ms, as observed for slowdowns in *Δche* (see Fig. 2b). This indicates that a flagellar motor can reverse its sense of rotation at least one order of magnitude faster than previously reported¹⁰. Furthermore, the occurrence of pausing in motors operating at normal load was confirmed for the first time.

Mutation(s) in the switch-complex genes increased the occurrence and severity of slowdowns, pause and reversals (Fig. 3). This suggests that the switch complex affects not only rotational direction but also torque generation and therefore rotation speed. Although it is possible that the Mot proteins, MotA and MotB, are the flagellar torque generator^{11,12} and the switch complex controls the state of Mot proteins, it seems more likely that the Mot proteins and the switch complex work together as a torque generator.

The temporal resolution of our method is high enough to detect the rapid fluctuations that should be closely related to the elementary processes of torque generation. More detailed analysis of rotation speed, which can be obtained by further processing the data we have collected or by using a position-sensitive detector to measure directly the displacement of the bright spots, should give us further insights into the mechanisms of torque generation and switching of rotation. □

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Solution structure of an unusually stable RNA hairpin, 5'GGAC(UUCG)GUCC

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HAIRPIN loops are important structural elements of RNA, helping to define the three-dimensional structure of large RNAs and providing potential nucleation sites for RNA folding and interaction with other nucleic acids and proteins¹⁻³. Little, however, is known about the conformation of RNA hairpins, most of what we know coming from transfer RNA crystal structures⁴ and from studies of DNA hairpins⁵⁻⁸. We report here the determination of the structure of a very stable and common RNA hairpin, 5'GGAC(UUCG)GUCC (loop nucleotides in parenthesis), by NMR spectroscopy. The sequence C(UUCG)G occurs very often in RNA⁹ and may be a nucleation site for RNA folding and a protein-binding site. A high-resolution structure for the hairpin was derived from interproton distances and scalar coupling constants determined by NMR. The loop is stabilized by a G·U base pair, with guanine in the *syn* conformation, a cytosine-phosphate contact and extensive base stacking. These findings and other structural features of the loop can explain the unusual stability of the hairpin and suggest why reverse transcriptase cannot read through the loop⁹, although it can transcribe through other kinds of RNA secondary structure.

The natural RNA hairpin sequence is 5'pppGGAC(UUCG)GUCC, but the mutant sequence 5'pppGGAC(UUUG)GUCC will also form a stable hairpin (Fig. 1) in 10 mM sodium phosphate (pH 6.7), as judged from gel filtration chromatography and ultraviolet absorption melting profiles¹⁰. The melting temperature (T_m) of the natural hairpin is $71.5 \pm 0.5^\circ\text{C}$ (which is about 20°C higher than the T_m predicted for hairpins with similar stem sequences¹¹ and tetrabase loops); for the mutant, T_m is $64.8 \pm 0.5^\circ\text{C}$. Both molecules were investigated by nuclear magnetic resonance (NMR) spectroscopy. We were able to assign all the imino and stem amino resonances, 10 out of 11 phosphorus resonances and all but a few non-exchangeable protons, including H5' and H5''. Assignments were achieved using several correlated experiments and NOESY^{12,13}.

Scalar coupling constants were measured in high-resolution correlated experiments (Fig. 2). Backbone torsion angles for the atoms O5'-C5'-C4'-C3'-O3' (β , γ , δ and ϵ) were estimated, with an uncertainty of $\pm(15^\circ-25^\circ)$, from the scalar couplings using semi-empirical Karplus equations^{14,15}. Torsion angles α and ζ on either side of a phosphorus were restricted to *gauche* ($-100^\circ \leq \alpha, \zeta \leq +100^\circ$) when the corresponding phosphorus resonance was not shifted downfield¹⁶. Inter-proton distances were determined using standard methods¹² from nuclear Overhauser effect

(NOE) build-up rates measured at short mixing times (60-200 ms).

A three-dimensional structure for the natural hairpin (Fig. 3) was obtained by distance geometry (DSPACE; Hare Research,

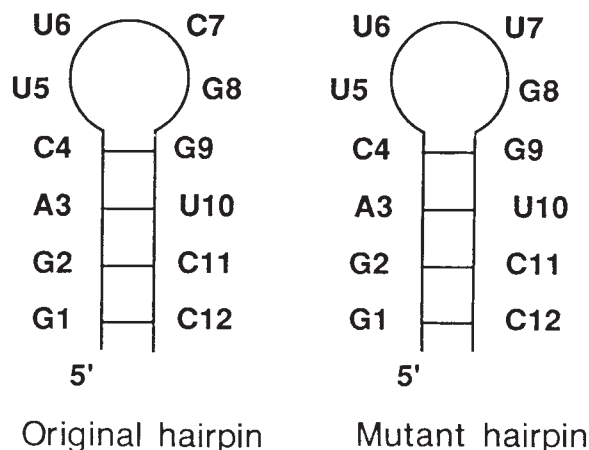


FIG. 1 Sequence and secondary structures of the two RNA hairpins. METHODS. RNA was synthesized *in vitro* using T7 RNA polymerase and synthetic DNA templates^{13,20}. Oligoribonucleotides were extracted with phenol after transcription and precipitated with ethanol, and then purified by electrophoresis on 20% polyacrylamide denaturing (7M urea) gels. After electroelution from the gel, the purified RNA was reprecipitated and dialysed against 10 mM phosphate buffer, pH 6.7.

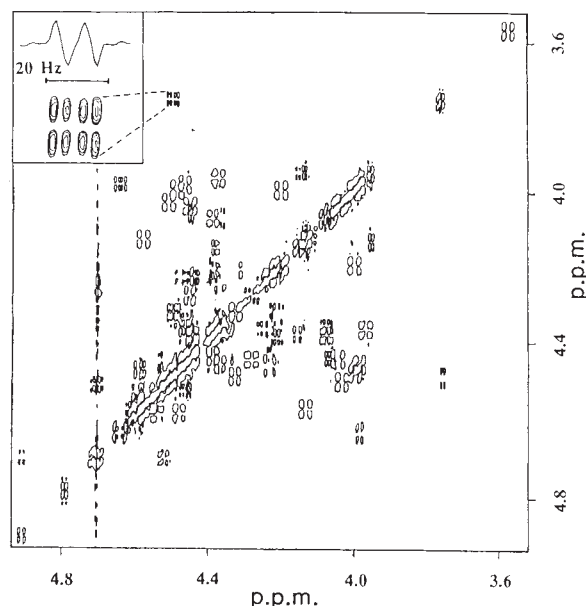


FIG. 2 ³¹P-decoupled, high-resolution (1 Hz per point) 2-quantum-filtered COSY¹² of the RNA hairpin (1.5 mM RNA in 10 mM sodium phosphate, pH 6.7, at 25°C). Only crosspeaks between sugar protons (H2', H3', H4', H5' and H5'') are shown; every isolated crosspeak has been identified and assigned. The H2'-H3' crosspeak for U₅ and a horizontal section through the crosspeak are shown expanded.

METHODS. About 5 mg purified RNA was dialysed against 10 mM phosphate buffer, lyophilized 3 times from 99.6% D₂O and suspended in 0.5 ml of 99.96% D₂O. All NMR spectra were taken on a GE GN-500 spectrometer (500 MHz for ¹H, 202 MHz for ³¹P). Acquisition time was 18-22 h for all two-dimensional NMR experiments. Data were processed using the program FTNMR (Hare Research, Inc.). Sugar protons were assigned, despite spectral overlap, by a combination of several homo- and heteronuclear correlated experiments (COSY, TOCSY, 2-quantum spectroscopy)^{12,13}. Assignments were made without any assumptions about conformation. ³¹P-¹H scalar couplings provided sequential connectivity (H3' → P → H4', H5', H5'') between sugar protons on neighbouring nucleotides^{21,22}, and ¹H-¹H couplings were used to identify the sugar spin systems.

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TABLE 1 Backbone torsion angles* of the hairpin

Angle	P5'-O5'	O5'-C5'	C5'-C4'	C3'-O3'	O3'-P3'	Glycoside	Pucker
	α	β	γ	ϵ	ζ	χ	$P^\dagger$
G ₁				-153	-66	-174	6
G ₂	-81	177	69	-164	-63	-171	9
A ₃	-84	-173	66	-164	-66	-159	20
C ₄	-99	-169	64	-163	-84	-146	31
U ₅	-92	172	61	-165	-87	-131	32
U ₆	-169	169	63	-80	-80	-172	189
C ₇	-90	-173	60	-83	82	-153	165
G ₈	73	180	177	-118	-67	43	18
G ₉	-44	129	89	-166	-65	-159	14
U ₁₀	-84	174	69	-166	-65	-157	15
C ₁₁	-86	179	70	-168	-66	-162	19
C ₁₂	-81	-176	62			-157	18
A-form‡	-68	178	54	-153	-71	-158	18

* The angles were obtained from one of the structures generated by distance geometry and constrained energy minimization. The structure with the best fit to the distance geometry constraints, and the lowest AMBER conformational energy after energy minimization, was chosen. Unusual angles are in bold face.

† Pseudorotation phase angle. C3'-endo sugar puckering corresponds to $P=18^\circ$, and C2'-endo to $P=162^\circ$.

‡ From fibre diffraction studies of A-form RNA¹⁹.

Inc.); no assumptions about conformation were made. We used conservative inter-proton distance constraints (four ranges of distances corresponding to strong, medium, weak and non-existent NOEs; see legend to Fig. 3), plus dihedral angle constraints based on scalar coupling constants. Six out of 25 trial structures converged to the point where the largest deviations from experimental constraints were less than 0.06 Å. All backbone torsion angles were within the range established from the

scalar couplings. The converged structures were refined by constrained energy minimization using the AMBER¹⁷ potential to improve van der Waals' and electrostatic contacts. Very low conformational energies were obtained with small changes from the distance geometry structures.

Several structural features of the loop were revealed by the NMR data and were confirmed in the high-resolution structure (see Fig. 4, and Table 1):

■ Stacking continues from both strands of the stem into the loop. Uracil U₅ stacks on the stem as in A-form RNA, whereas the stacking of guanine G₈ is unusual. The very strong aromatic to H1' NOE shows that G₈ is in the *syn* conformation; stacking is conserved at the expense of conformational changes in the backbone between G₈ and G₉. Large phosphorus-H5', H5'' couplings show that β of G₉ is *gauche*, not *trans*, and the G₈pG₉ phosphorus is downfield from the stem resonances. As g^+ and g^- conformers for β cannot be distinguished without stereospecific assignment of H5' and H5'', two distinct backbone conformations are consistent with the data. Either the C3'-O3'-P-O5' torsion angles (ϵ , ζ , α) are all *gauche*, and β is g^+ , or otherwise ϵ and α are *trans*, and β is g^- . Other parts of the hairpin and its overall folding are not affected by the two possible conformational arrangements at this location.

■ NOEs from U₅ and G₈ imino protons to imino and amino resonances of the C · G base pair closing the stem indicate the formation of a U · G base pair. The NOE from the G₈ imino to the proton H5 of U₅, and the downfield shift of the U₅ imino, support a reverse-wobble pair (Fig. 4c). But the NOE between the G and U imino is weak, and the G imino proton is not shifted downfield, as it would be if it were hydrogen bonded. In some distance geometry structures, the G · U pair was open towards the major groove, with the G₈ amino close to the C₇pG₈ phosphate. Only the U imino-G carbonyl hydrogen bond was

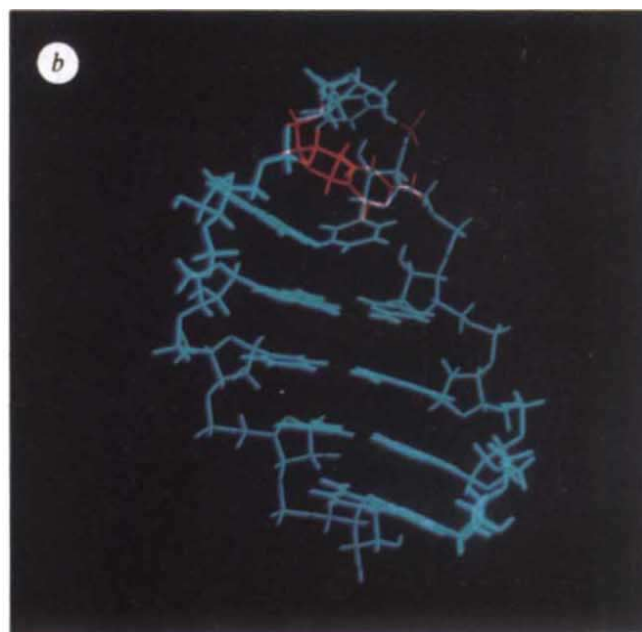
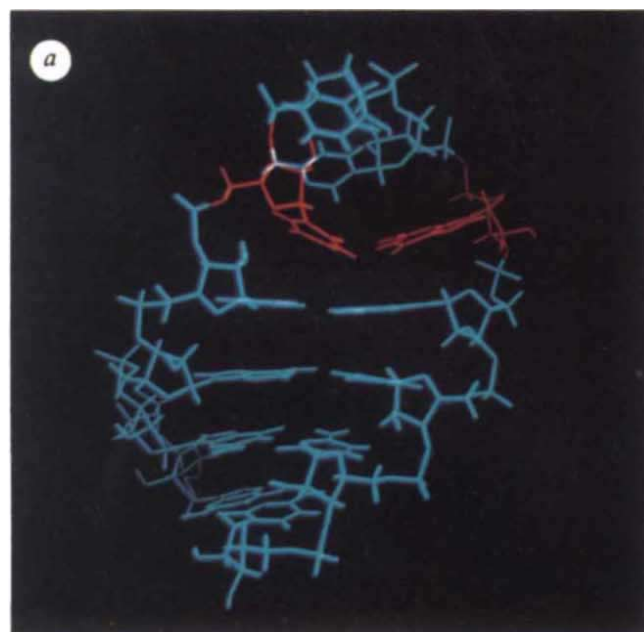


FIG. 3 Structure of the RNA hairpin from distance geometry followed by constrained energy minimization. *a*, View from the minor groove: the buckled U₅ · G₈ base pair is shown in red. *b*, Major groove view: C₇ and the U₅pU₆ phosphate are depicted in red. The C₇ amino-phosphate oxygen contact is indicated by the white bar.

METHODS. Interproton distances were obtained from NOE build-up rates, using pyrimidine H5-H6 distances (2.41 Å) as references. Two isolated H5'-H5'' crosspeaks were useful controls; the calculated H5'-H5'' distances (1.7 ± 0.2 Å) agree very well with crystal data (1.78 Å), suggesting that spin diffusion is small. We used 415 constraints in distance geometry; 125 intranucleotide interproton distances, 157 internucleotide interproton distances, 121 distances to define 86 backbone and sugar torsion angles, and

12 hydrogen bonds (11 for the stem, one for the G · U base pair). No conformational assumptions were introduced. Dihedral angle constraints were converted into distance constraints by using simple geometrical relations and standard bond lengths and bond angles¹⁹. NOESY crosspeaks between non-exchangeable protons were classified as strong, medium, weak or absent. Corresponding precision on distance constraints were 1.8-3 Å, 2-4 Å, 2.5-5 Å, and >4 Å, respectively. Precisions on distance constraints involving exchangeable protons were assumed to be 2-5 Å when NOEs were observed, and >4 Å for some NOEs that were clearly absent. Distance geometry structures were subject to constrained energy minimization using the AMBER¹⁷ potential with a distance-dependent dielectric constant, and ~150 constraints obtained from the distance geometry structures.

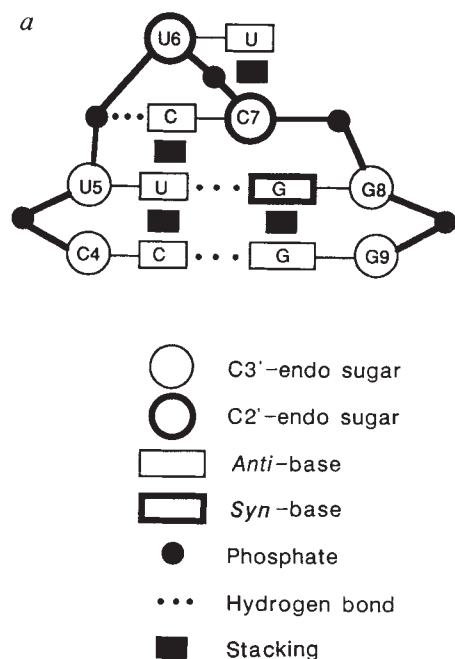


FIG. 4 *a*, Schematic diagram of the loop conformation. *b*, C_7 -amino hydrogen facing phosphate oxygen of U_5pU_6 ; base atoms and sugar protons for U_5 and U_6 are not shown. The unusual g^-t conformation for the $O3'-P-O5'$ torsion angles (ζ, α) of U_5pU_6 is stabilized by the amino-phosphate interaction. *c*, *Anti*- U_5 -*syn*- G_8 -base pair. The base pair is considerably buckled, probably to diminish the cross-strand distance to be spanned by the two loop nucleotides.

introduced as a distance geometry constraint. Thus, we cannot distinguish whether U_5 and G_8 form a reverse-wobble pair with two hydrogen bonds, or whether the base pair has one hydrogen bond with an amino-phosphate contact to stabilize the *syn* guanosine. The reverse-wobble base pair has lower AMBER conformational energy.

■ This hairpin has a loop of only two nucleotides, uridine U_6 and cytidine C_7 . Both adopt $C2'$ -endo (like B-form DNA) sugar pucker. This extends the backbone¹⁹ and helps bridge the stem. We have found this to be a general property of RNA hairpins with small loops.

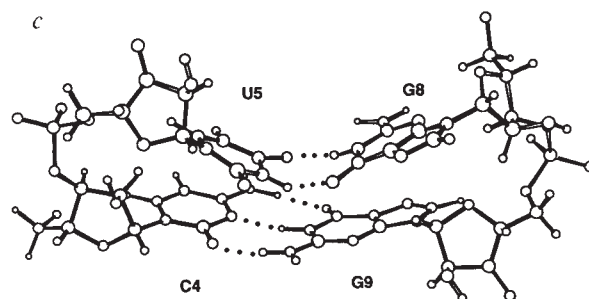
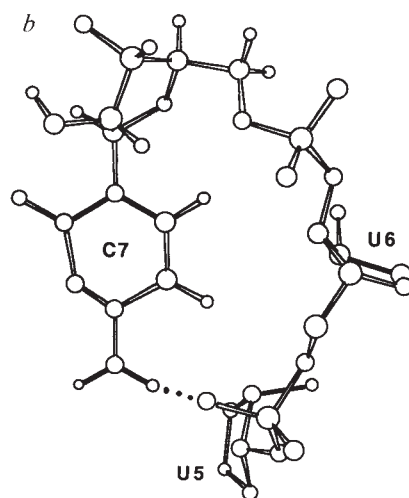
■ Unusual NOEs between U_5 and U_6 sugar protons require the torsion angle α , $P-O5'$, of U_6 to be *trans*, thus making the backbone turn sharply towards the opposite side of the stem. The downfield shift of this phosphorus resonance is consistent with the torsion angle α being *trans*.

■ The turn is stabilized by a favourable contact, probably a hydrogen bond, from the cytidine C_7 amino to a U_5pU_6 -phosphate oxygen. Strong NOEs from the cytidine C_7 proton H5 to both U_5 and U_6 sugar protons require the cytidine C_7 amino to be close to the phosphate linking U_5 and U_6 .

■ The C_7 nucleotide stacks on the $G \cdot U$ base pair.

The favourable cytidine C_7 amino-phosphate contact can explain why the mutant hairpin, in which this C is replaced by U, is less stable ($\Delta\Delta G$ at 37 °C is 1.5 ± 0.3 kcal mol⁻¹). NMR studies on the mutant show that there are small differences from the natural hairpin in chemical shifts, scalar couplings and NOEs. Thus, the structures are similar to each other. The substitution of U_7 for cytidine C_7 changes the amino group of C_7 into a carbonyl group. The different stability can be explained by the less favourable base-phosphate contact, and by different stacking on the $G \cdot U$ base pair.

The loop conformation agrees with the pattern of chemical modification on the four C(UUCG)G hairpin loops of *Escherichia coli* 16S ribosomal RNA¹⁸. Chemical probes strongly modified the U_6 imino, partially modified the C_7 nitrogen N3, and weakly modified U_5 and G_8 . In our structure, U_6 is outside, N3 of C_7 faces the solvent, and U_5 and G_8 iminos are protected.



Furthermore, uracil U_6 , not involved in specific interactions, is often mutated to A in rRNAs. Thus the proposed structure is likely to be similar to that of hairpins with the same sequence in intact 16S rRNA.

A hairpin loop of such compact shape and with many favourable interactions must, by itself, be energetically stable, and could provide nucleation sites for correct folding of rRNAs. The procession of reverse transcriptase may be blocked by this unusual structure, an intrinsically stable loop, although the enzyme can denature double-stranded RNA. Our results emphasize the role of base-phosphate interactions, which can be as specific as base-base interactions in determining RNA structure. □

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